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Method Development And Optimization For The Recovery Of Carbohydrates From A Microalga Species Of Chlorella Vulgaris By Combined Physical And Chemical Pre-Treatments

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METHOD DEVELOPMENT AND OPTIMIZATION FOR THE RECOVERY OF CARBOHYDRATES FROM A MICROALGA SPECIES OF *CHLORELLA VULGARIS* BY COMBINED PHYSICAL AND CHEMICAL PRE-TREATMENTS

by

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This thesis, submitted by William Hammann in partial fulfillment of the requirements for the Degree of Master of Science from the University of North Dakota, has been read by the Faculty Advisory Committee under whom the work has been done and is hereby approved.

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This thesis is being submitted by the appointed advisory committee as having met all of the requirements of the School of Graduate Studies at the University of North Dakota and is hereby approved.

Dean of the School of Graduate Studies

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Title	Method Development and Optimization for the Recovery of Carbohydrates from a Microalga Species of *Chlorella vulgaris* by Combined Physical and Chemical Pre-treatments
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Signature William Hammann

Date July 8th, 2019

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

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To my mom, Jean,

the best parent I could ever ask for.

ABSTRACT

A key focus of microalgae-based fuels/chemicals research and development has been on the lipids that many strains generate, but recent studies show that solely recovering these lipids may not be cost competitive with fossil-derived processes. However, if the carbohydrates can also be recovered and ultimately converted into useful chemical intermediates, this may improve the economics for microalgae-based sustainable product technologies.

In the present work, physical and chemical pre-treatments were performed on the *Chlorella vulgaris* microalgae strain to recover the carbohydrates from the biomass. A central composite design approach was used to optimize hydrolysis conditions including temperature, acid concentration, microalgae solid-to-liquid loading, and hydrolysis time. Results showed that the highest recovery of total carbohydrates obtained was 90 ± 1.1 wt% at 95% confidence with hydrolysis of 20 mL/g of ball-milled biomass performed in an autoclave at 120 $^{\circ}$C using 4 wt% sulfuric acid for 30 minutes. This represents a recovery of 40 wt% of the initial biomass weight. Sequential recovery of carbohydrates and lipids was also explored. Lipid recovery was maximized with pure methanol as a solvent, at a solid-to-liquid loading of 10 mL/$g_{biomass}$, at a temperature of 180 °C for 20 minutes in an autoclave. The highest recovery of total lipids was 71 ± 1.8 wt%, which represents 22 ± 0.9 wt% of the initial biomass weight. The sequential extraction of carbohydrates followed by lipids resulted in recovery of 60 ± 1.6 wt% of the initial biomass weight with 90% recovery of carbohydrates and 59% recovery of lipids. Even

though the recovery of total lipids was reduced, around 60 wt% of the biomass was made available for further transformations into fuels or other higher value chemicals, which is higher than current single product recovery strategies.

CHAPTER I
INTRODUCTION

The quest for clean and renewable energy sources for the future ranks as one of the most challenging problems facing mankind [1]. Currently, 80-88% of global energy consumption is derived from fossil fuels, such as petroleum, coal, and natural gas through combustion [2,3]. Extensive use of fossil fuels for power generation and as transportation fuel has resulted in high carbon dioxide emissions into the atmosphere and there is an urgent need to reduce this emission to mitigate additional detrimental impacts from the associated global warming [4]. In year 2010, the emission of CO2 was 110 billion metric tons and the emissions are forecasted to reach over 140 billion metric tons of CO2 in year 2035. The rate of global CO2 emission by various sectors from 1990 to 2035 is shown in Figure 1 [5]. High levels of CO2 in the atmosphere obstruct the flow of thermal radiation emitted from the earth's surface back into space that consequently causes the temperature of the earth to increase.

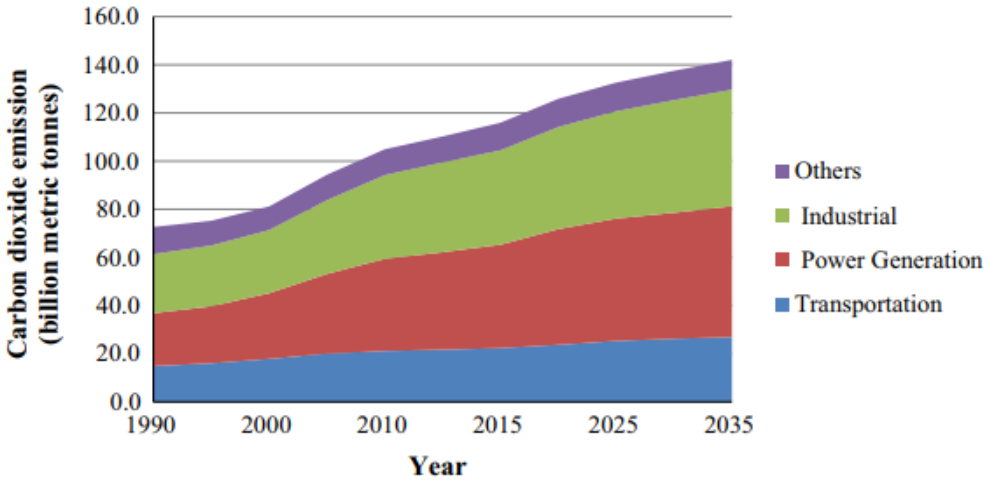

Figure 1: Global CO2 emission from various sectors from 1990 projected to the year 2035

While sustainable sources of energy are rapidly increasing, the current trajectory is insufficient due to the projected increase in the world's population, continued global industrialization and the increasing demand for transportation fuels [6]. Biomass is one of the most promising renewable resources and is currently used to generate first generation biofuels, such as biodiesel and bioethanol [7,8] as well as emerging second generation biofuels such as hydrotreated jet fuel. The term biofuel refers to solid, liquid, or gaseous fuels that are predominantly produced from biorenewable feedstocks [9].

Biofuels can be classified based on their production technologies: first generation biofuels (FGBs); second generation biofuels (SGBs); and third generation biofuels (TGBs) [10]. FGBs are economically viable and already produced at the industrial scale from terrestrial crops such as corn, soybean, and palm oil [11]. SGBs from biomass

residues, non-edible crops and wastes and TGBs including mainly microalgae, are emerging into the marketplace in recent years [12].

The advantages and challenges of using microalgae in biorefineries are summarized in Table 1. Cultivation is capable of year round production because growth can be done on open raceway ponds or in photobioreactors [13,14]. With respect to air quality maintenance and improvement, microalgae biomass production can effect biofixation of waste CO2 because for every 1 kg of biomass synthesized, about 1.8 kg of CO2 are consumed [14]. Microalgae are promising for the production of multiple components like lipids, carbohydrates, proteins and pigments that can be used as functional additives for cosmetic, chemical, and food products as well as for the production of biofuels [15,16].

In order to have an economically feasible production process intracellular component use should be maximized [17,18]. Selected components in microalgae can be targeted during the growth phase to accumulate significantly higher quantities by controlling key parameters such as light intensity, pH, temperature, inoculum size, and nutrient sources [19]. The main reasons the potential of microalgae has gained considerable focus in recent years are its ability to grow on non-arable land, fast growth and superior oil productivity compared to crop oils [20].

Table 1: Advantages and challenges of microalgae-derived biofuels

Advantages	**Challenges**
Capable of year round production [21]	Species selection must balance requirements for biofuel production and extraction [27]
Can be cultivated on non-arable land [22]	Potential for negative energy balance after accounting for requirements in water pumping, CO2 transfer, harvesting and extraction [28]
Growth rates can double their biomass in periods as short as 3.5 hours [14,23,24]	Limitations in scale-up leads to high capital cost [10]
Nutrients for cultivation can be obtained from wastewater [25]	High costs for dewatering [21]
Contain high amounts of valuable intracellular components [24]	Integrated processes for complete utilization of the biomass still need to be developed to be more cost effective and sustainable [29]
Composition can be modified by varying growth conditions [26]	

Microalgae's cultivation on non-arable lands can also be extended to growth on industrial wastewater streams and ponds [29-32]. Nutrients for microalgae cultivation (especially nitrogen and phosphorus) can be obtained from wastewater. Therefore, there is the dual potential of treating an industrial effluent while providing a growth medium for new fuel and chemical products [25]. One study showed that the *Chlorella Vulgaris* microalgae strain is capable of efficient nitrogen and phosphorus removal from Vilnius City wastewater by removing 87-93% of total nitrogen and up to 87% of total phosphorus

in the municipal wastewaters [33]. The cost of conventional removal of nitrogen and phosphorus is reported to be \$4.4 kg^{-1} N and \$3.05 kg^{-1} P removed [21]. The combination of saving from wastewater treatment and reduction of microalgae production costs is thus a win-win strategy when used for the production of energy or fuels [34].

On the other hand, one of the major disadvantages of microalgae for biofuel production is the low biomass concentration in the culture due to the limit of light penetration [10]. The low biomass concentration in combination with typically small size of microalgae cells makes the harvest of biomass relatively costly compared to traditional food crops resulting in a higher raw material cost. Therefore, on top of harvesting strategies to reduce costs, a species that allows a more complete utilization of the biomass is necessary in order to adopt commercial implementation of biofuels from microalgae.

Microalgae represent an enormous biodiversity from which about 40,000 species are already described or analyzed [35]. One of the most remarkable is the green eukaryotic microalgae *Chlorella Vulgaris*, which belongs to the following scientific classification: Domain: Eukaryota, Kingdom: Protista, Division: Chlorophyta, Class: Trebouxiophyceae, Order: Chlorellales, Family: Chloorellaceae, Genus: Chlorella, Specie: *Chlorella vulgaris* [30]. Martinus Willem Beijerinck, a Dutch research, first discovered it in 1890 as the first microalga with a well-defined nucleus [36]. *Chlorella* is a unicellular microalga that grows in fresh water and has been present on earth since the pre-Cambrian period 2.5 billion year ago and since then its genetic integrity has remained constant [37].

By the early 1900s, *Chlorella's* protein content of greater than 55 wt% attracted the attention of German scientists as an unconventional food source [30]. In the 1950s, the Carnegie Institution of Washington took over the study and managed to grow this microalga on a large scale for CO2 sequestration [38]. In more recent times, Japan has become the world leader in consuming *Chlorella* and uses it for medicinal purposes because it has been shown to have immune-modulating and anti-cancer properties [39-41]. Annual production of *Chlorella* reached 2000 tons (dry weight) in 2009, with Japan, Germany and Taiwan as the main producers [21]. *Chlorella* is ideal for mass production because it is remarkably resistant against harsh conditions and invaders [30].

Chlorella vulgaris, in particular, has been recognized as a potential feedstock for biofuel production due to its capacity to accumulate high levels of lipids and carbohydrates [14,42,43]. Lipid and carbohydrate contents increase along with biomass productivity during unfavorable growth conditions such as nitrogen and phosphorus limitation [44], high CO2 concentration [45], excessive exposure to light [46-49], excess of iron in the medium or an increase in temperature [50]. Cultivation techniques have been studied extensively in order to target biomass productivity regarding carbohydrates, lipids and protein content.

Lipids are a heterogeneous group of compounds that are defined not by their structure but rather by the fact that they are soluble in non-polar solvents and relatively insoluble in water [51]. *Chlorella* can reach 5-40 wt% lipids per dry weight of biomass depending on growth conditions [52]. The lipids are typically composed of long-chain

triacylglycerols (TAG) and represent a form of energy storage that is 2.25 times greater than carbohydrates on a weight basis [53]. TAGs can be converted to transportation fuels and chemicals by many emerging and developing processes. A strategy to reduce economic risk of such processes is to produce a diversity of higher value co-products in addition to transportation fuels [54]. Kubatova et al. [55] found that the non-catalytic cracking of canola and soybean oils led to the formation of 15-25 wt% of shorter-chain carboxylic acids (C2-C10) and 30 wt% linear alkanes. The market value of the C2 portion, represented as acetic acid, is comparable to transportation fuels and the C7-C10 products have many times the value of fuels [54].

Carbohydrates are the major products derived from photosynthesis and the carbon fixation metabolism (i.e. the Calvin cycle) [45]. Carbohydrates are either accumulated in the plastids as reserve materials such as starch, or become the main component of cell walls (e.g. cellulose, pectin, and polysaccharides) [56-58]. Cellulose is a structural polysaccharide with high resistance, which is located on the cell wall of *Chlorella vulgaris* as a protective fibrous barrier [59,60]. Both starch and cell wall polysaccharides can be easily converted to simple sugars that can be used as feedstock to produce ethanol or other chemical intermediates through microbial fermentation and decomposition processes [61-63]. *Chlorella* can accumulate a large amount of carbohydrates, up to 55 wt% of its dry biomass [21,56].

At the current state of microalgal-derived biofuel technologies, an algae-based process is not economically competitive with fossil and other renewable fuel processes.

One strategy is to generate higher value chemicals so that the biorefinery can be competitive [64].

The US Department of Energy has proposed a list of 12 potential biobased platform chemicals obtained by the screening of around 300 substances. Selection criteria were biomass precursors (carbohydrates, lignin, fats, and proteins), process platforms, building blocks, secondary chemicals, intermediates, products, and final application [65]. The reported platform chemicals can be produced from sugars biologically and/or chemically. The building-block chemicals can subsequently be converted into a multitude of high-value biobased chemicals and materials.

By producing multiple products, a biorefinery can take advantage of the differences in biomass components and intermediates and maximize the value derived from the biomass feedstock [66]. In order to fully utilize microalgae as a feedstock, both the lipids and carbohydrates must be efficiently recovered and purified. This body of work focused on determining an optimum method for the recovery of carbohydrates from the autotrophic *Chlorella vulgaris* microalgae strain. Building upon the work of colleagues at UND, the ability to sequentially recover carbohydrates followed by lipids was also assessed. Implementation of a combined process for the recovery of carbohydrates and lipids from the biomass would reduce the economic risk to adopt microalgae-based product technologies and help to mitigate global climate.

CHAPTER II
CARBOHYDRATE RECOVERY

The following was submitted as a journal article to the biochemical engineering journal. Supplemental information is provided in Appendix A.

Abstract

A key focus of microalgae-based fuels/chemicals research and development has been on the lipids that many strains generate, but recent studies show that solely recovering these lipids may not be cost competitive with fossil-derived processes. However, if the carbohydrates can also be recovered and ultimately converted into useful chemical intermediates, this may improve the economics for microalgae-based sustainable product technologies. In the present work, physical and chemical pre-treatments were performed on the *Chlorella vulgaris* microalgae strain to disrupt, convert and recover complex carbohydrates as simple sugars. A central composite design approach was used to optimize hydrolysis conditions including temperature, acid concentration, microalgae solid-to-liquid loading, and hydrolysis time. Results showed that the highest recovery of total carbohydrates obtained was 90 ± 1.1 wt% at 95% confidence with hydrolysis of 20 mL/g of ball-milled biomass performed in an autoclave at 120 °C using 4 wt% sulfuric acid for 30 minutes. We were able to identify that 92 wt% of the total carbohydrates in the extract solutions were a combination of simple sugars, which is ideal because they

will not require further hydrolysis prior to chemical transformations or fermentation processes.

2.1 Introduction

The cultivation and utilization of microalgae biomass as a source of renewable fuels and other chemicals has been an active area of research. Microalgae exhibits high productivity and high concentrations of valuable intracellular components, like lipids (fatty acid-based oils), proteins, and polysaccharides [67-70]. Microalgae as an alternative fuel source has many benefits including fast growth by having a doubling time of less than 24 hours, and storing energy in multiple forms [71]. Its biomass produces an energy yield that is 7-31 times greater per unit of cultivation area as compared to other biomass sources such as palm oil, corn and soybeans [10]. Microalgae can also be used for the treatment of waste-water, such as agro-industrial and domestic wastewaters [34,72,73]. Cultivation of microalgae in wastewaters allows nutrients to be captured and recycled, as well as conversion of wastewater organic matter as the carbon source to grow the biomass [74,75]. Using wastewater streams for microalgae growth, will not only reduce the need for further treatment of the water but is also ideal because the biomass will not be competing for land used for food based crops.

A main focus of microalgae-based fuels/chemicals research and development has been on the lipids that many strains of microalgae generate, but current research shows that solely recovering the lipids does not compete with fossil-derived processes [14,29].

When only the lipids are recovered, up to 60% of the biomass is wasted because it contains carbohydrates as glucans that can be further processed for energy production [56,62,76]. If the glucans can also be recovered and ultimately converted into useful chemical intermediates such as lactic and levulinic acid, microalgae-based sustainable product technologies will be more productive and potentially economically favorable.

Carbohydrates in microalgae are primarily contained in the cell wall in the form of cellulose and soluble polysaccharides, with additional carbohydrates located in the cell's plastids in the form of starch [30,68,77,78]. The composition of carbohydrates varies significantly based on the strain of microalgae because of environmental conditions such as light intensity, inoculum size, and nitrogen starvation periods during the growth phase [56,76,79].

In this study, research focused on the optimization of carbohydrate recovery from the *Chlorella vulgaris* microalga strain. *Chlorella vulgaris* accumulates 33-55% of its biomass as carbohydrates and the remaining components include lipids, proteins and other intracellular components [19]. Depending on growth conditions, up to 93% of the carbohydrates can be recovered as glucans [80-82]. High glucan recovery efficiency is highly desirable because glucans do not require further hydrolysis prior to biofuel processing.

A barrier to microalgae-based technology development is the ability to efficiently recover all sources of energy in the biomass. A challenge with the current state of

research in carbohydrate recovery is the length of time required to achieve recovery percentages over 90% of the total carbohydrates. Current research includes autoclaving hydrolysis and enzymatic hydrolysis that require upwards of 1 hour and 72 hours, respectively [83]. The use of microwave technology should increase rupture of the cell wall from the microwaves and expedite the time required for hydrolysis because of the rapid internal heating of the biomass. Therefore, microwave assisted hydrolysis should allow for a more rapid recovery of carbohydrates while still maintaining efficient recovery percentages.

The objective of this work was to identify optimum conditions for carbohydrate recovery through microwave- and temperature-assisted acid hydrolysis. Multiple pre-treatment methods were also investigated, such as ball-milling and sonication prior to acid hydrolysis, to determine the method that is most efficient and has the greatest recovery of carbohydrates. A benefit of acid hydrolysis for the recovery of carbohydrates is the acid simultaneously makes the carbohydrates available while also converting them into simple sugars [84].

2.2 Materials and Methodology

2.2.1 Materials

Freeze-dried autotrophic *Chlorella vulgaris* (80-120 mesh, Qingdao Sunrise Trading Co., Ltd., Qingdao, China) with the cell walls intact was obtained for this study. The freeze-dried biomass contained approximately 92% solids with the balance as

moisture, as determined by the NREL total solids in biomass determination protocol [85]. Experimentation with the microwave and respective analysis was completed at the University of Leeds in the United Kingdom, whereas the temperature-assisted and sonication pre-treatment work was completed and analyzed at the University of North Dakota in the United States. The ball-milled pre-treatment method was assessed in combination with both microwave- and temperature-assisted hydrolysis. The ball-milling pre-treatment was accomplished using a Retsch MP100 Planetary Ball Mill (Haan, Germany) for a total time of 15 minutes at a rate of 500 RPM to increase cell wall rupture.

2.2.2 University of Leeds Materials

Microwave-assisted hydrolysis was performed in a 1200W StartSYNTH MA084 Labstation (Sorisole, Italy). Features include a built-in focused IR sensor for non-contact temperature control, as well as a graphic display of time, temperature, and power, all of which can be modified to match desired sample conditions. Quartz sample vessels (cat. #: QB00045) placed inside a polytetrafluoroethylene reaction vessel (cat. #: DM00082A) were used. The hydrolysates were centrifuged in a Sigma 4-5L centrifuge (Osterode am Harz, Germany) at 1,200 RCF for 10 minutes and the supernatants were filtered using a single use 0.45-micron filter (Agilent-model, cat. #: 16555-K), and put into a 1.5 mL HPLC vial (Agilent-model, 5182-0864) for analysis. Liquid chromatography was conducted using a Thermoscientific Dionex UltiMate ACC3000 (Dionex Camberley, UK) coupled to a Shodex R-101 refractive index detector (Dionex Germering, Germany).

A Sigma-Aldrich Supercogel C610-H organic acid column (cat. #: 59320-U) was used in conjunction with a Shimadzu CTO-10AC column oven (Milton Keynes, United Kingdom) to sustain a temperature of 30 °C.

2.2.3 University of North Dakota Materials

Temperature-assisted hydrolysis was conducted in a Consolidated Sterilall Electricall heated double wall sterilizer type autoclave (Boston, USA). Liquid samples were then centrifuged using an IEC model HN-SII centrifuge (Needham HTS, USA) at 1,400 RCF for 10 minutes and filtered using an acrodisc syringe filter with a 0.2-micron nylon membrane (Pall Corporation cat. #: PN 4540), and put into a 2 mL HPLC vial (Agilent cat. #: 15337417) for analysis. Liquid chromatography was completed using an Agilent HPLC 1200 series with an Agilent Hi-Plex H organic acid column (cat. #: PL1170-68530, Stockport, UK) using a dilute sulfuric acid mobile phase (EMD Millipure Corporation H_2SO_4 98% for analysis EMSURE, Chicago, USA) coupled to a refractive index detector (Agilent model G1362A, Santa Clara, USA).

Sonication pre-treatment was performed in a Fisher Scientific 5.7 L ultrasonic bath model 15337417 (Pittsburgh, USA). Experiments were done at an ultrasonic power of 110 watts while sustained at a temperature of 40 °C.

A series of aqueous stock solutions were prepared containing five individual *Chlorella vulgaris*-specific sugars to create a calibration curve for each HPLC system

[86]. The stock solutions contained the following four monomeric carbohydrates, all purchased from Sigma-Aldrich with a purity of ≥99%: glucose (cat. #: G8270-5G), galactose (cat. #: G0750-5G), mannose (cat. #: M2069-5G-KC), arabinose (cat. #: A3131-5G) as well as L-(-)-fucose purchased from Thermo Fisher Scientific (cat. #: A16789, Heysham, United Kingdom). The stock solutions were prepared at known concentrations ranging between 0.1 and 4 mg/mL, which were then used for retention time evaluation and calibration of the individual monomeric carbohydrates.

2.3 Methodology

2.3.1 Microwave-assisted Acid Hydrolysis

A full central composite design of experiments with three replicates was conducted to determine optimized hydrolysis conditions for carbohydrate recovery. The main factors under investigation were temperature, sulfuric acid concentration, hydrolysis time, and solid-to-liquid loading. The levels for each factor are presented in Table 2. Factors that were not optimized during the process were microwave power output and the temperature ramp up time which were held constant at 1100 Watts and 10 minutes, respectively. The results from the experimental work were statistically analyzed using the Minitab software (NIST, v.18).

Table 2: Factors investigated in the design of experiments and their respective levels

Factors	Acid Concentration (wt%)	Hydrolysis Time (minutes)	Solid-to-Liquid Loading ($mL/g_{biomass}$)
Low	1.0	10	10:01
Center	2.5	15	15:01
High	4.0	20	20:01

Dried *Chlorella vulgaris* was weighed out as 1 gram samples and placed into quartz vessels. Each sample was subjected to different conditions in triplicate regarding sulfuric acid concentration, hydrolysis time, and solid-to-liquid loading based on the design of experiments schedule and inserted into the polytetrafluoroethylene reaction vessel and capped. The vessel was then attached and secured to the carousel inside the microwave. The microwave program consisted of 10 minutes of temperature ramp up time, the desired hold time at temperature, and a 10-minute cool down period.

After the microwave program had finished, the hydrolysate was removed from the quartz vessel and centrifuged for 10 minutes at 1,200 RCF. The supernatant was then collected, filtered and placed into a HPLC vial for analysis. The HPLC used an organic acid column with a mobile phase of 0.1% H_3PO_4 in deionized H_2O that had a flow rate of 0.5 mL/min at a pressure of 8,300 kPa, with a total run time of 45 minutes for the RI detector.

2.3.2 Temperature-assisted Acid hydrolysis

For experiments to study temperature-assisted hydrolysis, the optimized conditions for temperature, acid concentration, and solid-liquid loading that resulted from the design of experiments with the microwave-assisted hydrolysis experiments were used. Hydrolysis time was readdressed in this method because an autoclave requires longer temperature ramp up time and cool down compared to a microwave. Dried *Chlorella vulgaris* was weighed out as 500 mg samples and placed into pressure tubes with 10 mL of 4 wt% H_2SO_4. The length of time in the autoclave for hydrolysis was studied from a range of 20 to 90 minutes. After hydrolysis, each sample was centrifuged at 1,400 RCF, filtered and the liquid phase placed into HPLC vials for analysis. The HPLC used a Hi-Plex column with a mobile phase of 5 mM H_2SO_4 that had a flow rate of 0.6 mL/min at a pressure of 6,500 kPa, with a total run time of 45 minutes for the RI detector.

Two methods of pre-treatment were investigated to increase the recovery of carbohydrates prior to hydrolysis in an autoclave: 1) ball-milling the dried biomass and 2) sonicating the sample prior to the autoclave assisted hydrolysis. Ball-milling included grinding the biomass for 15 minutes at a rate of 500 RPM in a planetary ball mill. Sonication was conducted in an ultrasonic bath at a temperature of 40 °C for a length of 30, 60, or 90 minutes.

2.4 Results and Discussion

The main objective of this work was to identify optimum conditions for the recovery of carbohydrates from *Chlorella vulgaris* biomass by dilute sulfuric acid hydrolysis. Microwave- and temperature-assisted hydrolysis with biomass pre-treatment were considered to enhance the recovery of carbohydrates and this research included experiments for both of these methods. Optimization of experimental conditions was conducted in the microwave and the experimental method was then repeated in the autoclave so that the two methods could be compared.

Temperature showed a significant effect on the yield of carbohydrates. A screening study was completed over a temperature range of 100-140 °C prior to running the design of experiments (results not shown). The study resulted in minimal recovery of carbohydrates below a temperature of 120 °C because there was not enough energy to break down the biomass and facilitate the hydrolysis. At temperatures between 120-140 °C, degradation of carbohydrates into glucose derivative acids and other unknown components began to occur and the recovery dropped significantly. Therefore, the optimum temperature was determined to be 120 °C, which is similar to previous carbohydrate extraction research [87]. A temperature of 120 °C was held constant during the investigation of other factors in the design of experiments.

Table 3 summarizes the results obtained from the design of experiments with the microwave-assisted carbohydrate recovery and hydrolysis method. A total of 6 trials were

performed at the most optimum conditions for validation. Also, a total of 3 trials with ball-milled biomass were performed at optimum conditions to determine how the pre-treatment method effects carbohydrate recovery. Using the microwave-assisted hydrolysis method, 80 ± 1.6 wt% of the total carbohydrates were recovered from this particular strain of dried *Chlorella vulgaris* without pre-treatment and 81 ± 0.98 wt% after being ball-milled at a 95% confidence level. Ball-milling the biomass prior to the microwave-assisted hydrolysis proved to show no significant statistical improvement and is therefore an unnecessary additional energy requirement in the process.

Total carbohydrate composition of the dry biomass was determined following the NREL two-step acid hydrolysis protocol [88]. Previous work [86] suggests that glucose accounts for up to 76% of the total carbohydrates in the biomass, but in the present study, the glucose composition at the optimum conditions was around 61% with a slightly higher galactose composition than in previous work, as shown in Table 4. It is important to note, less than 10% of the carbohydrates are unknown longer chain polysaccharides that may require further hydrolysis prior to biofuel production. Therefore, even though the glucose yield is slightly lower than previously reported, the resulting carbohydrate solutions are still a very suitable feedstock for biofuel processes.

Table 3: Complete central composite design matrix done in triplicate at 95% confidence for optimization of total carbohydrate recovery by the microwave-assisted hydrolysis method

Std Order	Acid Concentration (wt%)	Hydrolysis Time (Minutes)	Solid-to-Liquid Loading ($mL/g_{biomass}$)	Fraction of Total Carbohydrates Recovered (wt%)
1	1.0	10	10	50 ± 3.3
2	4.0	10	10	43 ± 4.6
3	1.0	20	10	39 ± 1.5
4	4.0	20	10	76 ± 2.3
5	1.0	10	20	55 ± 0.6
6	4.0	10	20	62 ±1.4
7	1.0	20	20	56 ± 0.4
8	4.0	20	20	80 ± 1.6
9	0.0	15	15	1.0 ± 0.1
10	5.0	15	15	54 ± 2.4
11	2.5	7	15	55 ± 0.7
12	2.5	23	15	53 ± 0.3
13	2.5	15	7	51 ± 0.7
14	2.5	15	23	54 ± 1.3
15	2.5	15	15	54 ± 0.4
16	2.5	15	15	56 ± 0.8
17	2.5	15	15	54 ± 0.4
18	2.5	15	15	52 ± 0.5
19	2.5	15	15	54 ± 1.1
20	2.5	15	15	55 ± 0.6

.

Table 4: Composition of total carbohydrates recovered from the Chlorella vulgaris biomass used in this study for the maximum total recovery case, number 8, table 2.

Sugar	Percent of Total Carbohydrates	Confidence Interval at 95%
Glucose	61	±3
Galactose	27	±0.9
Arabinose	1	±0.05
Unknown	6	±2

Examining the results in more detail, it was found that only one interaction parameter was significant within the bounds of the experiments, i.e., the interaction between acid concentration and hydrolysis time. The interaction is significant because the recovery of carbohydrates did not show a linear trend with increasing hydrolysis time or acid concentration. Instead, the trend showed slight curvature which required further investigation to bound the optimum time and concentration, as seen in Figure 2. The significance of the interaction was expected because as acid concentration is increased, less time should be necessary to achieve complete hydrolysis. For the other two interactions, an increase in the condition of either factor will lead to an increase in carbohydrate recovery. Therefore, the levels of each factors were extended beyond the design of experiments in order to bound the optimum conditions.

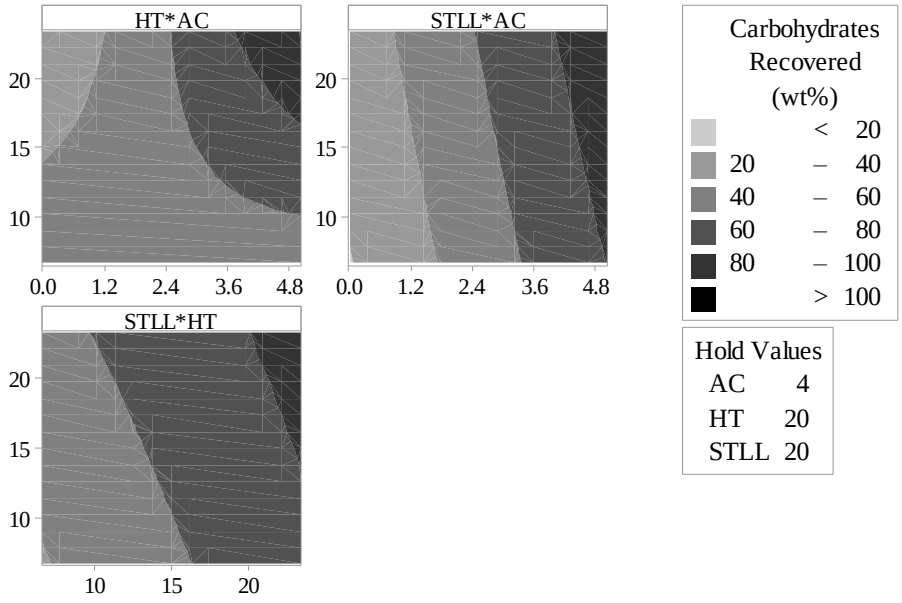

Figure 2: Central composite design contour plots showing the effect of interactions between significant factors from a study of microwave assisted carbohydrate recovery and hydrolysis. AC – Acid concentration (wt%); HT – Hydrolysis time (minutes); STLL – Solid-to-Liquid loading (mL/$g_{biomass}$)

The concentration of sulfuric acid used for extraction and hydrolysis was studied within a range of 1-10 wt% sulfuric acid and showed a significant effect on the recovery of carbohydrates. The recovery of carbohydrates increased with an acid concentration up to 4 wt% acid and then began to decrease. Therefore, the optimum concentration for hydrolysis of carbohydrates from the biomass is around 4 wt%, as shown in Figure 3.

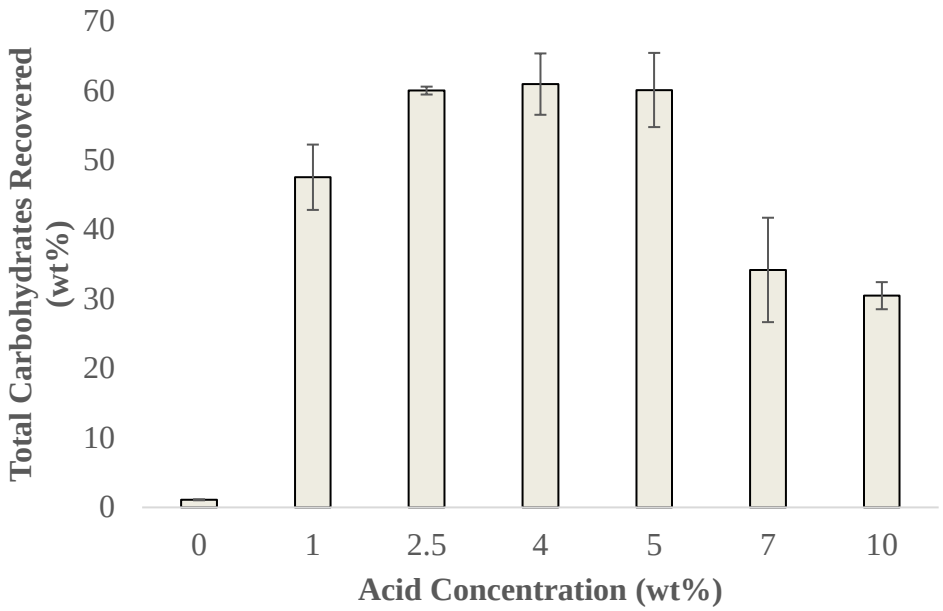

Figure 3: Total carbohydrate recovery versus sulfuric acid concentration during microwave-assisted hydrolysis

The hydrolysis time was varied from 6-40 minutes and significant changes in results occurred when varying this parameter as well. The recovery of carbohydrates increased until the 20-minute mark, where it then began to decrease linearly. Figure 4 presents this trend and shows that the optimum hydrolysis time is around 20 minutes.

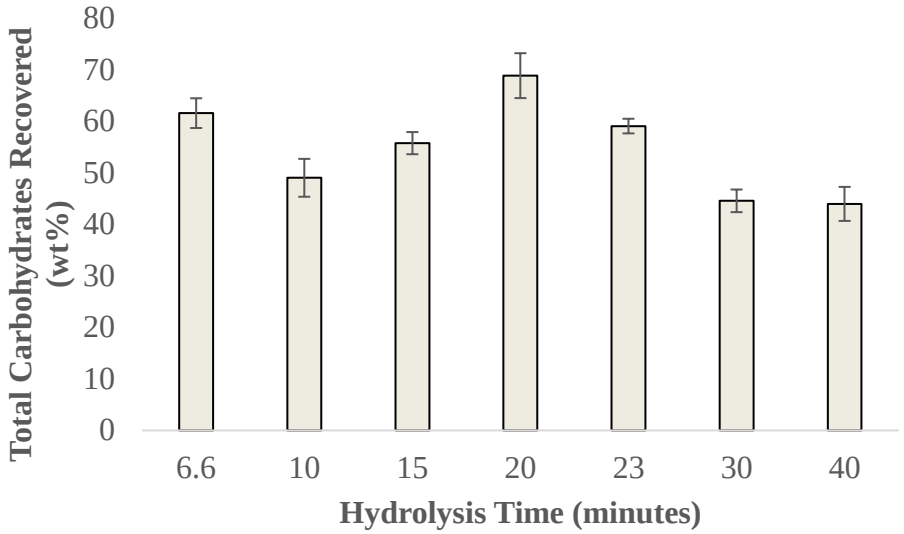

Figure 4: Optimization chart comparing the total recovery of carbohydrates versus hold time during hydrolysis

The solid-to-liquid loading was studied over the range of 6-30 mL/$g_{biomass}$. The yield of carbohydrates increased up to 20 mL/$g_{biomass}$ and then began to decrease. From this it can be seen that the optimum solid-to-liquid loading for efficient hydrolysis of carbohydrates from the biomass is around 20 mL of solvent per gram of biomass, as shown in Figure 5.

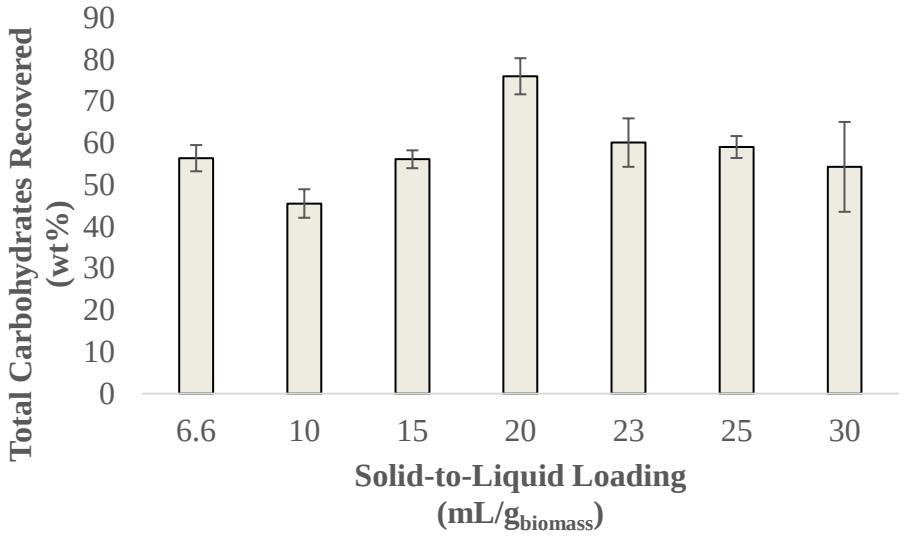

Figure 5: Optimization chart comparing the total recovery of carbohydrates versus solid-to-liquid loading

Optimum conditions (temperature = 120^{o}C, H_2SO_4 concentration = 4 wt%, hydrolysis time = 20 minutes, solid-to-liquid loading = 20 mL/$g_{biomass}$) from the design of experiments for microwave-assisted hydrolysis were then used in the temperature-assisted hydrolysis work with the exception of the hydrolysis time. The hydrolysis time was readdressed for the autoclave because the temperature ramp up time is different compared to the microwave. The hydrolysis time was studied from 20-90 minutes to determine the optimum length of time for a complete hydrolysis. The results of these experiments are shown in Table 5.

Table 5: Optimization of hydrolysis time for temperature-assisted hydrolysis in an autoclave

Hydrolysis Time (minutes)	Fraction of Total Carbohydrates Recovered (wt%)	Confidence Interval at 95%
20	87	±1
30	90	±1
60	82	±0.5
90	84	±2

The optimum hydrolysis time was determined to be around 30 minutes. Below the 30-minute mark resulted in a lower recovery of carbohydrates because a complete hydrolysis could not be achieved. Above the 30-minute mark also resulted in a lower carbohydrate recovery, as some of the carbohydrates likely started to degrade into other unwanted or unknown by-products.

Prior to hydrolysis, *Chlorella vulgaris* biomass was physically pre-treated by grinding the biomass in a ball-mill and/or sonicating the samples. The ball-milling process was not optimized in this study while sonication was studied from 30-90 minutes at a temperature of 40 °C.

In general, the sonication pre-treatment with or without ball-milling provided no significant statistical improvement and is therefore an unnecessary additional energy requirement in the process. Ball-milling followed by a 30-minute temperature-assisted acid hydrolysis resulted in the highest total carbohydrate recovery of 90 ± 1.1 wt% at 95% confidence. The next best result from obtained by the microwave-assisted acid

hydrolysis method with a recovery of 80 ± 1.6 wt% of the total carbohydrates at 95% confidence. When the samples were not ball-milled prior to temperature-assisted hydrolysis, total carbohydrate recovery reached 71 ± 2.3 wt% at 95% confidence. Thus, there were fairly significant differences in recoveries with each method of hydrolysis and additional pre-treatment, as presented in Figure 6.

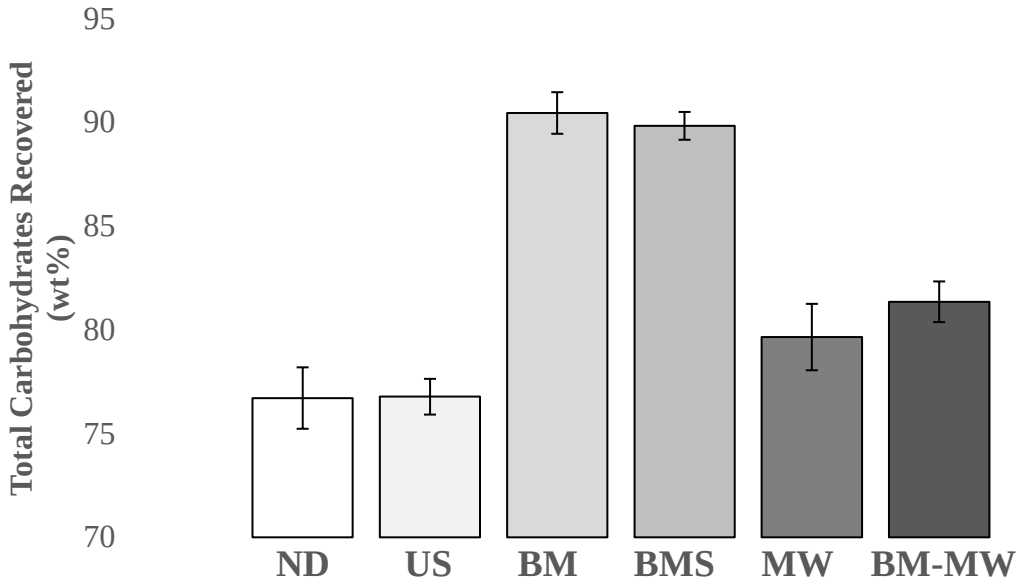

Figure 6: Total carbohydrates recovered for different pre-treatment methods. ND – no disruption; US – ultrasonication; BM – Ball-milled; BMUS – Ball-milled and ultrasonication; MW – microwave; BM-MW – Ball-milled and microwave

Previous studies suggest the carbohydrates in *Chlorella's* biomass are primarily stored in the form of starch prior to hydrolysis, which can be partially destroyed and lost when ball-milling the biomass [89]. The amount of lost carbohydrates was determined by

NREL total carbohydrate determination before and after ball-milling the biomass. The biomass samples of *Chlorella vulgaris* contained 44 ± 4 wt% of the biomass as carbohydrates, and approximately 10% were destroyed during the ball-milling pre-treatment process. Table 6 compares the composition of the total carbohydrates extracted based on the hydrolysis method followed. The composition of solely temperature-assisted acid hydrolysis without pre-treatment resulted in the highest concentration of unknown longer chain carbohydrates due to the lack of increased rupture from either ball-milling or microwaves. Therefore, even though some of the carbohydrates are destroyed in the ball-milling process, there was still a significant enough increase in recovery and reduction of unknown carbohydrates for this to be a viable pre-treatment method.

Table 6: Composition of total extracted carbohydrates at 95% confidence for microwave- and temperature-assisted acid hydrolysis with and without pre-treatment

Sugar	**Microwave Method**	**Ball-Milled Autoclave Method**	**Autoclave Method Without Pre-treatment**
Glucose	61 ± 1	58 ± 3	53 ± 0.4
Galactose	27 ± 3	25 ± 0.9	23 ± 1
Arabinose	1 ± 0.5	6 ± 0.02	4 ± 1
Fructose	-	3 ± 0.2	3 ± 0.7
Unknown	6 ± 2	8 ± 1	16 ± 0.9

Identification and quantification of the carbohydrate solutions were performed using a HPLC with a refractive index detector and an organic acid column. A minor issue with this approach is that the retention times for galactose and mannose are sufficiently similar such that they co-elute and are reported as a single peak. Previous work has concluded the presence of mannose in *Chlorella vulgaris* biomass is less than 2 wt% of the dry weight [86]. Therefore, this peak has been reported herein as galactose in Table 6. Also, up to 17 wt% of the carbohydrate composition has been reported as unknown longer chain sugars that require further work to identify.

2.5 Conclusion

In this study, methods for carbohydrate recovery by microwave- and temperature-assisted hydrolysis were further investigated to optimize the hydrolysis conditions for *Chlorella vulgaris* microalga. We conclude that a ball-milling pre-treatment released more of the carbohydrates contained in the cell wall and allowed for a higher recovery in the temperature-assisted acid hydrolysis in an autoclave, but was not necessary in the microwave. By optimizing the sulfuric acid concentration and solid-to-liquid loading, we were able to lower the typical time required in an autoclave while still maintaining a comparable recovery percentage. The optimized conditions for temperature-assisted acid hydrolysis with a ball-milled pre-treatment resulted in the recovery of 90 ± 1.1 wt% at 95% confidence of the total carbohydrates, which is higher than traditional single-step

recovery methods. Using a refractive index detector, the HPLC was able to identify approximately 92 wt% of the total carbohydrates in the extracted solutions were a combination of simple sugars, which is ideal because they will not require further hydrolysis prior to biofuel processing.

Acknowledgments

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CHAPTER III
COMBINED CARBOHYDRATE AND LIPID RECOVERY

3.1 Introduction

The production of fuels and chemicals from microalgae lipids remains one of the most sought after alternatives for fuels and chemicals but there are several challenges that remain [90,91]. Recovery of microalgae lipids is a multistep process which includes cultivation, harvesting, lipid extraction, and purification [92]. Many of the processes have a relatively high input cost that makes the process uneconomical, which is a major challenge to commercial microalgae-derived fuel production [93]. Shifting the focus from a single product strategy to integrated biomass processing for the recovery of carbohydrates in addition to lipids may help to develop a profitable venture [91].

Reports on extraction of individual intracellular components from microalgae are available in an abundance. However, to our best knowledge studies on identification and optimization of the sequential extraction of lipids and carbohydrates has not be documented. However, efforts have been made to use alternative low to medium energy consuming processes for lipid and carbohydrate recovery such as pulse electric field, ionic liquids, and surfactants [94,95]. These techniques are still under development and subject to further research before they could be optimized for commercial implementation [96].

Similar to carbohydrate recovery, cell disruption is a key parameter for increasing lipid extraction efficiency [97]. Mechanical pre-treatment of algae to disrupt the cell wall enhances solvent/lipid contact [98] and allows for easier recovery of the intracellular lipids, resulting in rapid and increased efficiencies in lipid extraction [97,99-101]. Typically, cell disruption not only improves access to stored lipids but also releases protein and carbohydrates [102]. Karemore et al. [103] investigated various pre-treatment methods and their effect on lipid recovery (Figure 7). Many of the methods are similar to pre-treatment methods for enhanced carbohydrate recovery. For further details on each method please reference Appendix A.

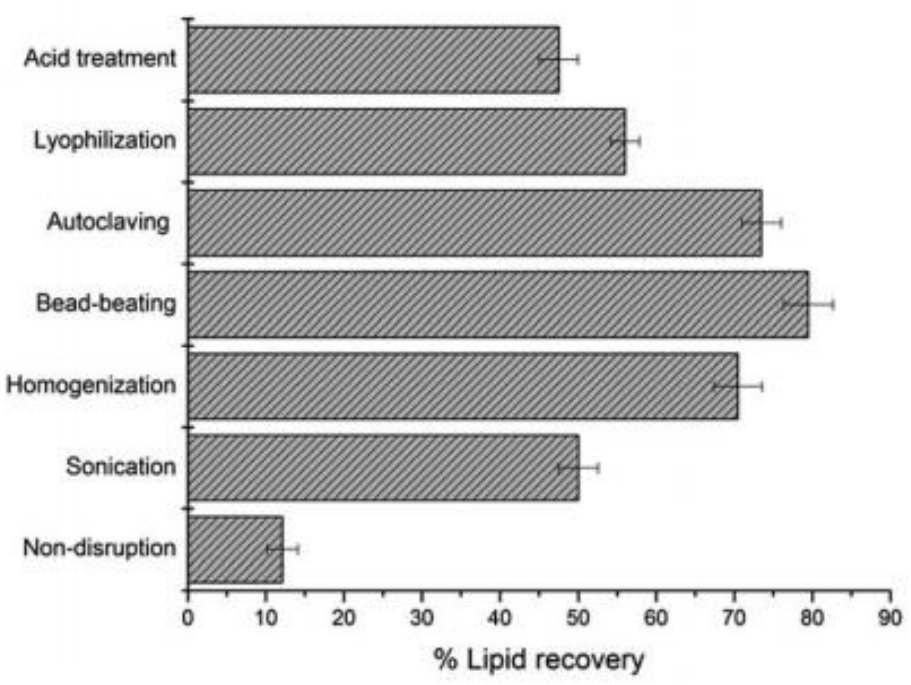


Figure 7: Effects of various cell disruption methods on total lipid recovery [103]

A well-documented method used to extract lipids from microalgae at the laboratory scale is Soxhlet extraction using hexane [104]. However, this approach has several disadvantages in terms of commercial viability [105]. First, the cell walls of microalgae are made up of a highly complex matrix of polysaccharides intercalated with proteins [106-108], which has a high chemical resistance to non-polar solvents. Second, hexane is incapable of extracting lipids stored in lipid droplets, as it cannot cross the (protein bound) polar phospholipid-membrane [105]. On the other hand, polar solvents such as methanol/chloroform cross the phospholipid barrier [109] by diffusion and extract these lipids (the Bligh & Dyer method [110]).

This body of work focuses on the sequential extraction of carbohydrates and lipids from *Chlorella vulgaris*. Carbohydrate recovery is performed using optimized methods described in chapter II. For lipids, this work relies on the results from two contemporary graduate students, Ian Foerster and Jasmine Oselik, who have been studying lipid extraction methods and conditions. Their full results will be published separately. Based on their results, we have performed lipid recovery by physically pre-treating the biomass by ball-milling followed by solvent extraction with methanol at elevated temperatures.

3.2 Materials and Methodology

Freeze-dried autotrophic *Chlorella Vulgaris* (80-120 mesh, Qingdao Sunrise Trading Co., Ltd., Qingdao, China) with the cell walls intact was obtained for this study.

The dried biomass was ground in a Retsch MP100 Planetary Ball Mill (Haan, Germany) at a rate of 500 RPM for a time of 10 minutes

Lipids were extracted at temperatures ranging from 120 to 200 °C in a Blue M Stabil-Therm oven (Blue Island, USA) with methanol as a solvent, purchased from Fisher Scientific at histological grade (cat#: A4335-22, Fair Lawn, USA). A solid-to-liquid loading of 10 $mL/g_{biomass}$ was used for each sample with an extraction time of 20 minutes at temperature. The samples were allowed to cool to room temperature and then were vacuum filtered onto pre-weighed filter paper. The liquid phase, including the methanol and extracted lipids, were inserted into a pre-weighed ThermoScientific 16 mL clear glass vial (cat#: B7999-4, Rockwood, USA) and placed into the oven at 50 °C overnight to evaporate all of the residual methanol. The residual biomass and filter paper was also placed into the oven at 50 °C to dry off any residual methanol remaining in the biomass.

3.3 Results and Discussion

The main objective of this work was to identify optimum conditions for the sequential recovery of lipids and carbohydrates from *Chlorella vulgaris* biomass. Foerster and Oselik have shown that using methanol at a solid-to-liquid loading of 10 $mL/g_{biomass}$ with a 20-minute extraction time provided the highest recovery of lipids from among a suite of candidate solvents. The temperature during the extraction process still requires optimization because it shows a significant effect on the yield of lipids [98]. Aguirre and Bassi et al. suggest there is a significant extraction efficiency of lipids from

Chlorella vulgaris when the temperature is increased above 110 °C and the optimum within the range of 110-200 °C [111]. Therefore, a study was completed over a temperature range of 120-200 °C to determine the optimum temperature for lipid recovery.

Significant changes in lipid recovery occurred when varying the extraction temperature. The recovery of lipids increased until a temperature of 180 °C where it began to decrease. Figure 8 presents this trend and shows that the optimum temperature for lipid extraction is around 180 °C. Using the complete optimized conditions (temperature = 180 ^{o}C, extraction time = 20 minutes, solid-to-liquid loading = 10 $mL/g_{biomass}$) resulted in a total lipid recovery of 71 ± 1.8 wt%, which represents 22 wt% of the initial biomass weight at 95% confidence.

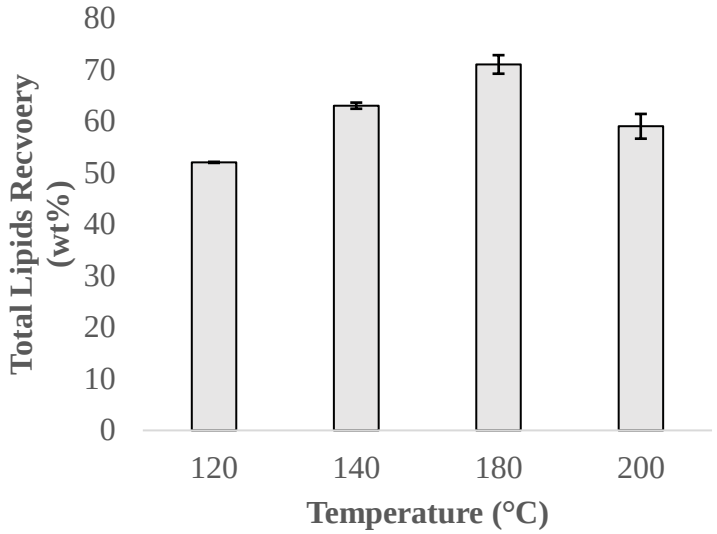


Figure 8: Optimization chart comparing the total recovery of lipids versus extraction temperature

The yields obtained for separately extracting lipids and carbohydrates from *Chlorella vulgaris* were found to be 71 ± 1.8 wt% of the total lipids and 90 ± 1.1 wt% of the total carbohydrates in the biomass at 95% confidence, respectively. These values provide an upper limit of the recoveries that could be expected from a sequential extraction method.

In the case of secondary carbohydrate recovery, extraction of carbohydrates from the lipid extracted biomass resulted in a significant decrease of total carbohydrate recovery. The recovery of carbohydrates from the lipid extracted biomass was 68 ± 2.8 % of the total carbohydrates initially in the biomass (30 wt% of the initial biomass). Up to 30% of the total carbohydrates initially in the biomass are lost during the pre-treatment and lipid extraction processes. However, up to 98 ± 1.3% of the carbohydrates that

remained in the biomass after the lipid extraction step were recovered, an 8% increase in recovery efficiency compared to the primary extraction of carbohydrates.

The lipid extracted biomass makes the carbohydrates more available because the lipids contained in the cell wall are removed allowing better access to the carbohydrates during hydrolysis. Figure 9 shows a simplified mass flow diagram for the loss and recovery of carbohydrates throughout the sequential extraction process. By sequentially recovering lipids followed by carbohydrates, 47 ± 3.1 wt% of the initial biomass weight is made available for further fuel or higher value chemical transformation processes.

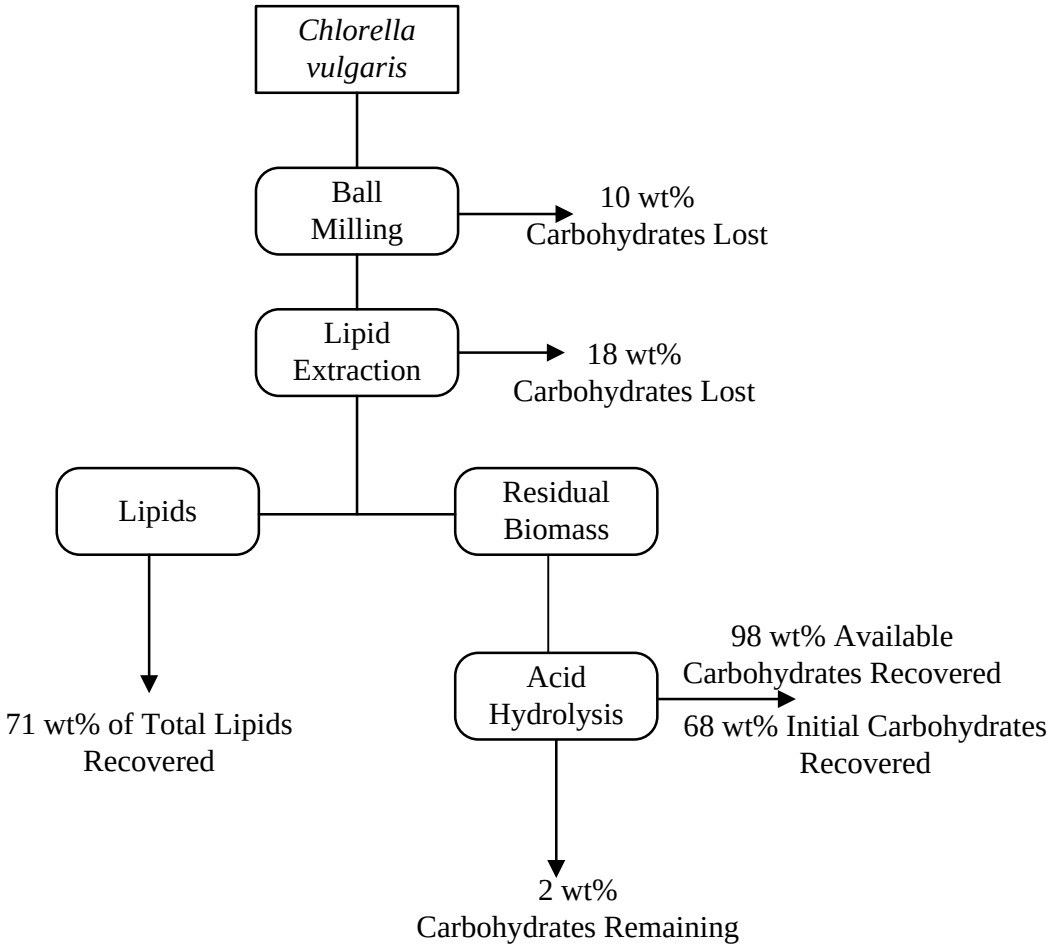


Figure 9: Mass flow diagram showing loss of carbohydrates during each step of the process and recovery of available and initial total carbohydrates

Multiple attempts were made to recover the carbohydrates that were lost during lipid extraction such as hexane/water wash, hexane/water wash followed by acid hydrolysis, and acid hydrolysis of residual oils. Both of the hexane/water wash methods recovered negligible carbohydrates that still remained in the oil. The acid hydrolysis on the residual oils showed a recovery of 50 ± 1.3 wt% of the carbohydrates that were lost

during the lipid extraction process, results are shown in Appendix E. The procedure was a simple screening study to see if the carbohydrates could be recovered and will require further work to optimize this method. However, even though some of the carbohydrates can be recovered through acid hydrolysis, this may be destroying or changing the composition of the oil and could make it unsuitable for further use. To our best knowledge, there are no published works that have looked into recovering lost carbohydrates during the lipid extraction process, so it may be advantageous to research this further.

In the case of secondary lipid recovery, the yield of total lipids decreased from 79 wt% to 59 ± 1.5 wt% (a loss of around 4 wt% of the initial biomass weight). Even though the recovery of total lipids was reduced, around 60 wt% of the biomass was made available for further transformations into fuels or other higher value chemicals, which is higher than current single product recovery strategies. By sequentially recovering carbohydrates followed by the lipids, 60 ± 1.5 wt% of the initial biomass weight is made available for further fuel or higher value chemical transformation processes.

3.4 Conclusion

Based on our work to date**,** sequential recovery should start with carbohydrate extraction and hydrolysis followed by lipid extraction. Our current best conditions for lipid extraction and recovery, was using methanol as a solvent, at a solid-to-liquid loading of 10 $mL/g_{biomass}$, at a temperature of 180 °C for 20 minutes in an autoclave. The

highest recovery of total lipids was 71 ± 1.8 wt%, which represents 22 ± 0.9 wt% of the initial biomass weight.

The residual carbohydrate-lean biomass after hydrolysis can be used as the feedstock for lipid recovery through solvent extraction at elevated temperatures. Up to 91 wt% of the total carbohydrates initially in the biomass can be recovered by the optimized acid hydrolysis method described in the previous chapter. The secondary total lipid recovery decreased by 20 wt% compared to lipids as the primary extraction product. Even though the total lipid recovery was reduced, the sequential extraction of carbohydrates followed by lipids resulted in recovery of 60 ± 1.6 wt%, which provides a much higher overall yield of platform lipids and sugars that can be used for further transformations into fuels or other higher value chemicals than from single product recovery strategies.

CHAPTER IV
SUMMARY AND CONCLUSIONS

A full central composite design of experiments study was used to optimize the conditions for carbohydrate recovery through microwave-assisted acid hydrolysis. The design of experiments showed that a temperature of 120 °C, hydrolysis time of 20 minutes, solid-to-liquid loading of 20 $mL/g_{biomass}$, and a sulfuric acid concentration of 4 wt% provided the highest recovery of total carbohydrates at 80 ± 1.6 wt%.

Optimum conditions resulting from the design of experiments were then used in the temperature- assisted hydrolysis work with the exception of hydrolysis time. The hydrolysis time was readdressed for the autoclave because the temperature ramp up time is different compared to the microwave. The optimum hydrolysis time was determined to be around 30 minutes. Prior to hydrolysis, *Chlorella vulgaris* biomass was physically pre-treated by grinding the biomass in a ball-mill and/or sonicating the samples. In general, the sonication pre-treatment with or without ball-milling provided no significant statistical improvement and is therefore an unnecessary additional energy requirement in the process. Ball-milling followed by a 30-minute temperature-assisted acid hydrolysis resulted in the highest total carbohydrate recovery of 90 ± 1.1 wt% at 95% confidence. By optimizing the sulfuric acid concentration and solid-to-liquid loading, we were able to lower the typical time required in an autoclave while achieving a higher recovery of total carbohydrates than traditional single-step recovery methods in the literature. Using a

refractive index detector, the HPLC was able to identify approximately 92 wt% of the total carbohydrates in the extracted solutions were a combination of simple sugars, which is ideal because they will not require further hydrolysis prior to biofuel processing.

Lipid recovery was maximized with pure methanol as a solvent, at a solid-to-liquid loading of 10 $mL/g_{biomass}$, at a temperature of 180 °C for 20 minutes in an autoclave. The highest recovery of total lipids was 71 ± 1.8 wt%, which represents 22 ± 0.9 wt% of the initial biomass weight.

Carbohydrates and lipids were sequentially recovered through a dilute acid hydrolysis and solvent extraction with a single process of physical cell disruption. The residual carbohydrate extracted biomass was used as the feedstock for lipids recovery through a solvent extraction with methanol at elevated temperature. The sequential extraction of carbohydrates followed by lipids resulted in recovery of 60 ± 1.6 wt% of the initial biomass weight with 90% recovery of carbohydrates and 59% recovery of lipids. Even though the recovery of total lipids was reduced, around 60 wt% of the biomass was made available for further transformations into fuels or other higher value chemicals, which is significantly higher than current single product recovery strategies.

Current work was all completed using freeze-dried biomass as a feedstock, but I recommend investigating the use of wet biomass. Freeze-drying the biomass involves a significant amount of energy to complete, and by eliminating this step in addition to recovering up to 60 wt% of the biomass could have the potential to make the process economically feasible. Also, contrary to previous research, the highest carbohydrate

recovery was obtained by simply the temperature-assisted acid hydrolysis. Therefore, further investigation into the microwave-assisted hydrolysis is necessary to determine if additional carbohydrates are being destroyed in the process and resulting in a lower recovery of carbohydrates due to the heating mechanism or the additional microwaves.

While this body of work shows the extraction process as batch steps, a continuous process could be developed or modeled to allow easier industrial scale-up of this technology. Since the key steps of the process solely require solvents and elevated temperatures, a reactor could be designed at the required temperature and allow for sufficient residence time to achieve efficient recoveries.

APPENDIX A

CARBOHYDRATE RECOVERY SUPPLEMENTAL INFORMATION

Detailed Experimental Setup

A planetary ball mill was used to grind the freeze-dried *Chlorella vulgaris* for a total time of 15 minutes at 500 RPM. For the microwave-assisted hydrolysis, approximately one gram of ground biomass was weighed in triplicate and inserted into quartz reaction vessels to be tested. 20 mL of 4 wt% sulfuric acid was added to the quartz reaction vessel and vortexed to ensure even mixing. The reaction vessel was then inserted into the polytetrafluorethylene reaction chamber, capped and secured to the carousel inside the microwave. The microwave program allotted 10 minutes for temperature ramp-up time, 20 minutes at a temperature of 121 °C, and a 10-minute cool down period. After the 40-minute microwave program had finished, the quartz reaction vessel was removed from the microwave carousel and the reaction chamber. The solution was emptied into a centrifuge tube and centrifuged at 1,200 RCF for 10 minutes. The liquid phase was decanted into a separate container and the solid was capped and placed into the freezer for storage. Approximately 1.5 mL of the liquid sample was filtered through a single-use 0.45-micron filter, and placed into a HPLC vial to be used for analysis.

The temperature-assisted hydrolysis followed a similar method to the microwave-assisted hydrolysis but include a few minor differences. One gram samples run in triplicate were weighed and placed in quartz pressure vessels to be tested. 20 mL of 4

wt% sulfuric acid was added to the quartz pressure vessel and vortexed. The vessel was then inserted into an autoclave set at a temperature of 121 °C. The autoclave took approximately 15-20 minutes to reach temperature and was held at temperature for 30 minutes. After the reaction was complete, the autoclave was depressurized and allowed to cool for approximately 30 minutes. Samples were removed from the autoclave, added to centrifuge tubes and centrifuged at 1,400 RCF for 10 minutes. Liquid samples were decanted into a separate container and the solids were capped and placed into the freezer for storage. 500 mL of each sample was filtered using a 0.2-micron nylon filter and placed into a HPLC vial for analysis. Also, 500 mL of deionized water was added to each sample to dilute the acid prior to analysis on the HPLC.

Method Selection

In order to recover carbohydrates from *Chlorella*, a disruption technique on the biomass must be carried out since most carbohydrates are entrapped within the cell wall, or intracellularly as energy storage in the form of starch [112,113]. Pre-treatments have been viewed as one of the most crucial and expensive processing stages in biomass conversion to fermentable sugars [114]. Research and development of various pre-treatment processes have great potential for improving the recovery of carbohydrates in addition to lowering costs. Despite of the many cell disrupting methods that have been previously developed in the literature for microalgae cell wall disruption, a common pre-treatment has not been identified that can treat most of the different microalgae species

[83]. Therefore, it is quite difficult to compare pre-treatment methods because results will be different based on different microalgae strains, growth conditions, and techniques that are employed during the process.

Saccharification is typically the rate limiting step in biofuel production using lignocellulosic (FGBs and SGBs) or microalgal biomass (TGBs) that contain a cellulose source [56]. While the process of saccharification of microalgae is similar to that of lignocellulosic materials, the lack of lignin present in the biomass simplifies the pre-treatment process because the cell wall is less rigid [115]. In general, pre-treatment and extraction methods are categorized as chemical, biological and physical [116]. Table 7 presents advantages and disadvantages to current methods of carbohydrate recovery from biomass. Further details of each method is described in the following subsections.

Among each of the reviewed methods, chemical and physical methods are currently the most efficient and include promising technology for industrial scale-up. Combinations of different pre-treatment have been also considered but not studied as extensively compared to single-step approaches. This body of work includes a robust investigation of optimizing carbohydrate recovery through microwave- and temperature-assisted hydrolysis combined with chemical, ball-milling and ultrasonication pre-treatment techniques.

Classification	Methods	Advantages	Disadvantages	Ref.
Chemical	Acid/Alkali	Low energy input, operates at industrial scale	Requires disposal of acid/alkali after extraction, carbohydrate degradation	[117,118]
	Ionic Liquid	Low cost	Still in their infancy, issues of over toxicity	[119-121]
Biological	Enzymes	Effective cell wall hydrolysis, high selectivity, carbohydrate bioactivity not affected	High cost of enzymes, longer treatment time, enzymes must be disposed of after use	[98]
Physical	Ball-Milling	Effective cell wall disruption, rapid extraction	Varied efficiency across species, high energy input and maintenance costs	[98]
	Microwave	Effective cell wall disruption and excellent recovery of bioactives, relatively low energy input, fast heating, reduced solvent usage	Generates heat, high maintenance cost, difficult to scale-up	[98]
	Ultrasonication	Effective cell wall disruption, minimal maintenance cost, no hazardous substances required	High operation costs and energy input	[98]
	Autoclave	Low maintenance cost	High energy input, not suitable for pigments, slower heating	[122,123]
	Pulsed Electric Field	High selectivity, mild treatment, carbohydrate bioactivity not affected, relatively low energy input	Still in its infancy	[68,124]

Table 7: Benefits and limitations of current pre-treatment methods for cell wall disruption

Acid Pre-treatment

Chemical pre-treatment processes have been successfully proven for various types of biomass including: corn [125], switchgrass [126], sugar cane [127] and straw [128]. The chemicals applied in the pre-treatment process are typically either hydrochloric or sulfuric acid, which are everyday industrial chemicals carrying minimal toxicity in their applied concentrations [78]. Acid pre-treatment is typically performed at dilute concentration because the use of concentrated acid is less attractive for ethanol production due to the formation of fermentation inhibiting compounds [129]. In the case of *Chlorella vulgaris*, it is known that the complex carbohydrates are entrapped in the cell wall of the microalgae [56,130,131], and must be released and converted into simple sugars in order for further processing into fuels or other high value chemical intermediates [132]. During the acid pre-treatment process, various conditions influence the total amount of sugars that can be released such as temperature, process time, solid-to-liquid loading, and acid concentration [78].

Alkaline Pre-treatment

The effect that some bases have on lignocellulosic biomass is the basis of alkaline pre-treatments, which can be very effective depending on the lignin content of the biomass [129]. Alkaline pre-treatments increase cellulose digestibility and are more effective for lignin solubilization, exhibiting minor cellulose and hemicellulose solubilization compared to acid or hydrothermal processes [133-135]. A major benefit to

alkali pre-treatment is that it can be performed at room temperature and it is described to cause less sugar degradation than an acid pre-treatment [134,136,137]. However, as with any pre-treatment method, possible loss of sugars and production of inhibitory compounds must be taken into consideration in optimization for the final desired products.

The alkaline pre-treatment method has been reported multiple times in the literature for various lignocellulosic biomass systems, but very minimal work has been reported for microalgal biomass [138]. Since microalgal carbohydrates are mostly entrapped in the cell wall, the pre-treatment process is required to free and breakdown complex carbohydrates into simple sugars for further down-stream processing [78]. Huran et al. investigated the alkaline pre-treatment on microalgal biomass for the first time by using sodium hydroxide [138]. With this method of pre-treatment, Huran was able to obtain a high recovery of glucose from the biomass meaning there exists a potential for further development of an alkali pre-treatment for enhanced carbohydrate recovery in non-lignocellulosic biomass.

Biological Pre-treatment

Enzymatic saccharification processes, involving the use of cellulases, amylases and glucoamylases, are widely used to hydrolyze microalgae to obtain sugars [56,139]. Harsh pre-treatment, such as acidic or alkaline pre-treatment is not necessary during this method, making it easier and cheaper to saccharify microalgae-based cellulose when

compared with lignocellulosic materials because of the absence of lignin and hemicellulose [56]. Different enzymes are used in the hydrolysis step and the process is influenced by numerous factors including cellulose crystallinity, substrate surface area, cell wall thickness, porosity, mass transfer, and hemicellulose or lignin contents [129]. Since microalgae have been reported to have no lignin composition [140], it can be categorized as a cellulosic based material and the cellulase enzyme could be applied to hydrolyse microalgal biomass. The cellulose-hydrolysing enzymes (cellulase) are obtained from fungi, bacteria or protozoans through cellulolysis of cellulosic materials [139]. The components catalyse the cellulosic materials in three different steps: (1) the endoglucanases break down the non-covalent interactions within the crystalline structure of cellulose; (2) the exoglucanases hydrolyse the individual cellulose fibers into simple sugars and the cellobiohydrolases attack the chain ends producing cellobiose; and (3) the -glucosidases release glucose monomers by hydrolysing the disaccharides and tetrasaccarides of cellulose [141,142]. Therefore, glucose will be the end result of enzymatic hydrolysis by cellulase.

In general, enzymatic hydrolysis offers advantages such as high recoveries, lower cost, low energy usage, no chemicals requirement, and relatively mild environmental conditions [98]. However, the main drawback to develop biological methods is the slow hydrolysis time compared to other techniques, which can take upwards of 72 hours [143]. Hernández et al. investigated a combined pre-treatment of dilute acid pre-treatment followed by an enzymatic hydrolysis [83]. With a combination of the two methods,

Hernández was able to achieve similar sugar recovery efficiencies, but with a much shorter enzymatic hydrolysis step because of the increase in cell wall rupture from the acid pre-treatment.

Ball-milling

Mechanical pre-treatment of microalgae to disrupt the cell wall and enhance the efficiency of the intracellular components recovery process by increasing the contact surface area between the desired component and the solvent [98]. Disruption of the cellular wall allows for an easier recovery of components in addition to decreasing the process time required [97]. Ball-milling is a process that works to disturb the extracellular wall of microalgae by grinding and agitation of the cells on a solid surface [144]. The two main factors that require consideration during ball-milling are the residence time and milling speed [145]. Each will have a major effect because if the biomass is left too long or grinded at too high of a rate, components in the biomass can begin to disintegrate and will decrease the recovery of products [146]. The main advantages of using ball-milling as a pre-treatment are the simplicity, rapidness of the method, reproducibility of results, and relatively high effectiveness [17,147].

Microwave

Microwave-based pretreatment can be considered a physicochemical process since both thermal and non-thermal effects are often involved. Pretreatments were carried out by immersing the biomass in dilute chemical reagents and exposing the slurry to

microwave radiation for residence times ranging from 5 to 20 min [148]. Microwaving causes rapid alignment and realignment of dipoles in a polar solvent, resulting in heat generation, which can disrupt cell wall structures and break down the carbohydrates present in microalgae [149]. Microwave assisted extraction from microalgae is one of the simplest methods and most effective amongst other extraction methods [97]. Due to simplicity and effectiveness, microwave technology is more suitable to large scale use compared to other methods [150]. The rapid extraction time, high heating rates, low operating costs, environmentally friendly nature, lesser solvent requirements, high product purity and high efficiency make it an attractive method for microalgae lipid recovery [83,98,129].

Ultrasonication

Ultrasonication is another mechanical method that can be used for pre-treatment of microalgae prior to carbohydrate extraction. During ultrasonication, the biomass is exposed to high intensity ultrasonic waves, creating tiny cavitation bubbles around the cells [151]. The bubbles then collapse and emit shockwaves that disrupt the cell walls causing the carbohydrates to be more available for further processing [152]. There are some contradictions in the literature regarding scale up. Halim et al. [150] noted that this technique is moderately suitable for scale up whereas Mercer and Armenta [144] stated that ultrasound maybe difficult for upscale. In spite of the minor research on ultrasound

pretreatment from lignocellulose, some researchers have also shown that saccharification of cellulose is enhanced efficiently by ultrasonic pretreatment [153].

In general, the addition ultrasonication does show an improvement of carbohydrate recovery compared to single-step methods, but more importantly it provides the benefit of opening up the surface of solid substrates to other methods like enzymatic or acid hydrolysis, similar to ball-milling, such that a better recovery can be achieved with a combination of the two. Pre-treating microalgae biomass using ultrasound has several advantages which include: reduced extraction time, less solvent requirement, higher yields as a result of easier cell penetration, the biomass does need to be dry and easier release of intracellular components to the bulk of the solvent [154]. Chemat et al. [155] and Wang and Weller [156] stated that ultrasound assisted extraction can be operated at low temperatures (less thermal denaturation of biomolecules) and is much more economical compared to other conventional extraction methods.

Autoclave

Autoclaving microalgae biomass is a form of thermal treatment operating at temperatures ranging from 100-200 $^{\circ}$C [157,158]. High thermal stress causes the cell walls to rupture forcing the release of the intracellular components [150]. Autoclaving cells at high temperatures over a short duration can reduce the degradation of the desired product [159]. The effectiveness of autoclaving treatment on different microalgae spices varies as a result of different cell wall structures that can be tough and unaffected by

autoclave disruption techniques [160]. Therefore, similar to ultrasonication, autoclave methods are typically combined with other methods such as acid, alkaline, or enzymatic hydrolysis.

Pulsed Electric Field

In the past decade, pulsed electric field (PEF) has been claimed to be a promising mild technique able to induce the permeability of the microalgal cells by electroporation and to enhance the release of intracellular components [15]. PEF processing involves the application of repetitive short duration pulses (from several nanoseconds to several milliseconds) of high intensity electric fields to a biological material placed between two electrodes in either batch or continuous flow treatment chamber [161]. As a result, PEF induces an increase in the permeability of the cell membranes by electroporation that facilitates the release of intracellular components [162]. At the current state of the research, PEF treatment seems not able to sufficiently disintegrate the algal cells to release carbohydrates at yields comparable to the benchmark ball-milling [70]. Therefore, further development of the PEF method should be investigated before it is considered a viable alternative.

APPENDIX B

SEQUENTIAL RECOVERY EXPERIMENTAL SETUP

A planetary ball mill was used to grind the freeze-dried *Chlorella vulgaris* for a total time of 15 minutes at 500 RPM. One gram samples of the ground biomass were weighed and inserted into quartz pressure vessels. One gram samples were weighed and placed in a quartz pressure vessel to be tested. 20 mL of 4 wt% sulfuric acid was added to the quartz pressure vessel and vortexed. The vessel was then inserted into an autoclave set at a temperature of 121 °C.

The autoclave took approximately 15-20 minutes to reach temperature and was held at temperature for 30 minutes. After the reaction was complete, the autoclave was depressurized and allowed to cool for approximately 30 minutes. Samples were removed from the autoclave, added to centrifuge tubes and centrifuged at 1,400 RCF for 10 minutes. The liquid phase was decanted into a separate container and 5 mL of ultrapure water was added to the centrifuge tube, shaken, and centrifuged at 1,400 RCF for another 10 minutes. The liquid was decanted into the same container with the initial liquid phase extract and the process was repeated one more time to wash the solids free of all residual carbohydrates. The residual biomass was then placed back into the pressure vessel to be prepared for lipid extraction. 10 mL of methanol was added to the pressure vessel and vortexed to ensure complete mixing. The vessel was then inserted into an autoclave set at 175 °C. The autoclave took approximately 15-20 minutes to reach temperature and was

held at temperature for 20 minutes. After the reaction was complete, the autoclave was depressurized and the samples were allowed to cool to near room temperature.

The mixture was vacuumed filtered using a pre-weighed filter and the vessel was rinsed with an additional 6 mL of methanol to remove all residual biomass from the vessel. The collected liquid was placed into a pre-weighed container. The container of liquid and filter with residual biomass were dried in a drying oven overnight at 50 °C until all solvent had evaporated. The weight of the container and filter after dying were measured and recorded to determine the total lipids recovered and the weight of residual biomass.

APPENDIX C

HPLC ANALYSIS

The supernatant from each experiment was filtered using a 0.45 (University of Leeds) or 0.2 micron filter (University of North Dakota) prior to being placed into a 2 mL LC vial. Exactly 500 mL of the filtered sample was combined with 500 mL of deionized water to dilute each sample in order to raise the pH of the solution. The LC vial was capped using a crimping tool followed by being vortexed for homogenization.

A series of aqueous calibration standards were prepared containing five individual *Chlorella Vulgaris*-specific sugars to create a calibration curve for each HPLC system [27]. The stock solutions contained the following monomeric carbohydrates, all purchased from Sigma-Aldrich with a purity of ≥99%: glucose, galactose, mannose, and arabinose. Also present in the solutions was L-(-)-fucose purchased from Thermo Fisher Scientific. Calibration standards were prepared by measuring 200 mg of each sugar accurately to .1 mg. The mixture of sugars was then dissolved in 50 mL of deionized water to have a concentration of 4 mg/mL and vortexed to ensure homogenization. Serial dilutions were done from the 4 mg/mL solution to create the desired number of calibration and concentrations. Approximately 1 mL of each calibration standard was filtered, placed into an LC vial, and capped using a crimping tool.

The HPLC used a Hi-Plex column with a mobile phase of 5 mM H_2SO_4 that had a flow rate of 0.6 mL/min at a pressure of 6,500 kPa, with a total run time of 45 minutes for the RI detector.All calibration standards and samples were placed in the autosampler tray

for analysis. The run order was started with two blanks consisting of solely mobile phase to ensure a stable baseline and there was no residue remaining in the column from prior use. After the two blanks, calibration standards were injected starting with the most dilute one. Then each of the samples were injected, another blank and calibration standard were included if the sequence runtime would take more than a day. The sequence finished with two more blanks to flush the column and to ensure there is nothing coming through late from a previous sample. The method also included two needle wash cycles prior to each injection to make sure the needle was not contaminated from the previous sample. An example of the HPLC sequence used for each experiment is presented in Table 8, below.

Table 8: Example of an HPLC analysis sequence

Vial	Sample Name	Method Name	Sample Amount (µL)	Inj Volume (µL)
31	01_Blank	19-02-08_55C_0.6ML_45MIN_WH	1000	15
31	02_Blank	19-02-08_55C_0.6ML_45MIN_WH	1000	15
87	03_WH_CAL1	19-02-08_55C_0.6ML_45MIN_WH	1000	15
86	04_WH_CAL2	19-02-08_55C_0.6ML_45MIN_WH	1000	15
85	05_WH_CAL3	19-02-08_55C_0.6ML_45MIN_WH	1000	15
96	06_WH_CAL4	19-02-08_55C_0.6ML_45MIN_WH	1000	15
95	07_WH_CAL5	19-02-08_55C_0.6ML_45MIN_WH	1000	15
80	08_WH_S1	19-02-08_55C_0.6ML_45MIN_WH	1000	15
79	09_WH_S2	19-02-08_55C_0.6ML_45MIN_WH	1000	15
78	10_WH_S3	19-02-08_55C_0.6ML_45MIN_WH	1000	15
90	11_WH_S4	19-02-08_55C_0.6ML_45MIN_WH	1000	15
89	12_WH_S5	19-02-08_55C_0.6ML_45MIN_WH	1000	15
88	13_WH_S6	19-02-08_55C_0.6ML_45MIN_WH	1000	15
31	14_Blank	19-02-08_55C_0.6ML_45MIN_WH	1000	15
31	15_Blank	19-02-08_55C_0.6ML_45MIN_WH	1000	15
1	01_Blank	19-03-05_50C_0.6ML_45MIN_WH	1000	15
1	02_Blank	19-03-05_50C_0.6ML_45MIN_WH	1000	15
87	03_WH_CAL1	19-03-05_50C_0.6ML_45MIN_WH	1000	15
86	04_WH_CAL2	19-03-05_50C_0.6ML_45MIN_WH	1000	15
85	05_WH_CAL3	19-03-05_50C_0.6ML_45MIN_WH	1000	15
96	06_WH_CAL4	19-03-05_50C_0.6ML_45MIN_WH	1000	15
95	07_WH_CAL5	19-03-05_50C_0.6ML_45MIN_WH	1000	15
60	08_WH21_1	19-03-05_50C_0.6ML_45MIN_WH	1000	15
59	09_WH21_2	19-03-05_50C_0.6ML_45MIN_WH	1000	15
58	10_WH21_3	19-03-05_50C_0.6ML_45MIN_WH	1000	15
57	11_WH21_4	19-03-05_50C_0.6ML_45MIN_WH	1000	15
56	12_WH21_5	19-03-05_50C_0.6ML_45MIN_WH	1000	15
55	13_WH21_6	19-03-05_50C_0.6ML_45MIN_WH	1000	15
1	14_Blank	19-03-05_50C_0.6ML_45MIN_WH	1000	15
1	15_Blank	19-03-05_50C_0.6ML_45MIN_WH	1000	15
1	01_Blank	19-03-26_50C_0.6ML_45MIN_WH	1000	15
1	02_Blank	19-03-26_50C_0.6ML_45MIN_WH	1000	15

Table 8: Continued

87	03_WH_CAL1	19-03-26_50C_0.6ML_45MIN_WH	1000	15
86	04_WH_CAL2	19-03-26_50C_0.6ML_45MIN_WH	1000	15
85	05_WH_CAL3	19-03-26_50C_0.6ML_45MIN_WH	1000	15
96	06_WH_CAL4	19-03-26_50C_0.6ML_45MIN_WH	1000	15
95	07_WH_CAL5	19-03-26_50C_0.6ML_45MIN_WH	1000	15
50	08_WH22_10	19-03-26_50C_0.6ML_45MIN_WH	1000	15
49	09_WH22_11	19-03-26_50C_0.6ML_45MIN_WH	1000	15
48	10_WH22_12	19-03-26_50C_0.6ML_45MIN_WH	1000	15
47	11_WH22_13	19-03-26_50C_0.6ML_45MIN_WH	1000	15
46	12_WH22_14	19-03-26_50C_0.6ML_45MIN_WH	1000	15
45	13_WH22_15	19-03-26_50C_0.6ML_45MIN_WH	1000	15
44	14_WH22_16	19-03-26_50C_0.6ML_45MIN_WH	1000	15
43	15_WH22_17	19-03-26_50C_0.6ML_45MIN_WH	1000	15
42	16_WH22_18	19-03-26_50C_0.6ML_45MIN_WH	1000	15
41	17_WH22_19	19-03-26_50C_0.6ML_45MIN_WH	1000	15
1	18_Blank	19-03-26_50C_0.6ML_45MIN_WH	1000	15
1	19_Blank	19-03-26_50C_0.6ML_45MIN_WH	1000	15
1	01_Blank	19-05-13_40C_0.6ML_45MIN_WH	1000	15
1	02_Blank	19-05-13_40C_0.6ML_45MIN_WH	1000	15
20	03_WH_CAL1	19-05-13_40C_0.6ML_45MIN_WH	1000	15
19	04_WH_CAL2	19-05-13_40C_0.6ML_45MIN_WH	1000	15
18	05_WH_CAL3	19-05-13_40C_0.6ML_45MIN_WH	1000	15
17	06_WH_CAL4	19-05-13_40C_0.6ML_45MIN_WH	1000	15
16	07_WH23_01	19-05-13_40C_0.6ML_45MIN_WH	1000	15
15	08_WH23_02	19-05-13_40C_0.6ML_45MIN_WH	1000	15
14	09_WH23_03	19-05-13_40C_0.6ML_45MIN_WH	1000	15
13	10_WH23_04	19-05-13_40C_0.6ML_45MIN_WH	1000	15
12	11_WH23_05	19-05-13_40C_0.6ML_45MIN_WH	1000	15
11	12_WH23_06	19-05-13_40C_0.6ML_45MIN_WH	1000	15
30	13_WH24_01	19-05-13_40C_0.6ML_45MIN_WH	1000	15
29	14_WH24_02	19-05-13_40C_0.6ML_45MIN_WH	1000	15
28	15_WH24_03	19-05-13_40C_0.6ML_45MIN_WH	1000	15
27	16_WH24_07	19-05-13_40C_0.6ML_45MIN_WH	1000	15
26	17_WH24_08	19-05-13_40C_0.6ML_45MIN_WH	1000	15
25	18_WH24_09	19-05-13_40C_0.6ML_45MIN_WH	1000	15

Table 8: Continued

1	19_Blank	19-05-13_40C_0.6ML_45MIN_WH	1000	15
1	20_Blank	19-05-13_40C_0.6ML_45MIN_WH	1000	15
1	01_Blank	19-05-13_40C_0.6ML_45MIN_WH	1000	15
18	02_WH_CAL3	19-05-13_40C_0.6ML_45MIN_WH	1000	15
24	03_WH_25_01	19-05-13_40C_0.6ML_45MIN_WH	1000	15
23	04_WH_25_02	19-05-13_40C_0.6ML_45MIN_WH	1000	15
22	05_WH_25_03	19-05-13_40C_0.6ML_45MIN_WH	1000	15
21	06_WH_25_04	19-05-13_40C_0.6ML_45MIN_WH	1000	15
40	07_WH_25_05	19-05-13_40C_0.6ML_45MIN_WH	1000	15
1	08_Blank	19-05-13_40C_0.6ML_45MIN_WH	1000	15
1	09_Blank	19-05-13_40C_0.6ML_45MIN_WH	1000	15
100	01_Blank	19-05-20_68C_0.3ML_45MIN_WH	1000	20
100	02_Blank	19-05-20_68C_0.3ML_45MIN_WH	1000	20
20	03_WH_CAL1	19-05-20_68C_0.3ML_45MIN_WH	1000	20
19	04_WH_CAL2	19-05-20_68C_0.3ML_45MIN_WH	1000	20
18	05_WH_CAL3	19-05-20_68C_0.3ML_45MIN_WH	1000	20
17	06_WH_CAL4	19-05-20_68C_0.3ML_45MIN_WH	1000	20
30	07_WH24_01	19-05-20_68C_0.3ML_45MIN_WH	1000	20
29	08_WH24_02	19-05-20_68C_0.3ML_45MIN_WH	1000	20
28	09_WH24_03	19-05-20_68C_0.3ML_45MIN_WH	1000	20
100	10_Blank	19-05-20_68C_0.3ML_45MIN_WH	1000	20
100	11_Blank	19-05-20_68C_0.3ML_45MIN_WH	1000	20
100	01_Blank	19-05-20_68C_0.3ML_45MIN_WH	1000	20
100	02_Blank	19-05-20_68C_0.3ML_45MIN_WH	1000	20
90	03_WH_CELLO	19-05-20_68C_0.3ML_45MIN_WH	1000	20
89	04_WH_GLU	19-05-20_68C_0.3ML_45MIN_WH	1000	20
88	05_WH_GAL	19-05-20_68C_0.3ML_45MIN_WH	1000	20
87	06_WH_MAN	19-05-20_68C_0.3ML_45MIN_WH	1000	20
86	07_WH_ARA	19-05-20_68C_0.3ML_45MIN_WH	1000	20
85	08_WH_FUC	19-05-20_68C_0.3ML_45MIN_WH	1000	20
100	09_Blank	19-05-20_68C_0.3ML_45MIN_WH	1000	20
100	10_Blank	19-05-20_68C_0.3ML_45MIN_WH	1000	20

APPENDIX D

HPLC DATA PROCESSING

The area of each sugar peak was determined using Agilent ChemStation software based on the deviation from the baseline created by the mobile phase. Table 9 shows each sugar with its respective retention times for each column used for analysis. Figure 10 and Figure 11 show a chromatogram from a temperature-assisted acid hydrolysis sample for both the Agilent Hi-Plex and CARBOSep column, respectively. The large peak at the beginning is sulfuric acid, which can be omitted by neutralizing the sample prior to analysis but was not done in this work. Calibration standards were prepared with known concentrations to create a curve that would allow for calculation of concentration based off the area under each peak, as shown in Figure 12 and Figure 13. Even though the same standards were used for each column, you can see that Figure 13 has six peaks compared to the five in Figure 12 because the H-Plex column was unable to separate glucose and galactose such that they appeared as a single peak. Figure 14 shows an example of the constructed calibration curve from the known standards and areas reported by the HPLC. Equations were created for each sugar such that the area could be transformed into concentrations.

Table 9: Sugar retention times for each column used in this study

	Agilent Hi-Plex	CARBOSep
Sugar	**Retention Time (minutes)**	
Glucose	11.5	26.1
Galactose	12.1	31.2
Mannose	12.1	37.6
Arabinose	13.2	34.8
Fucose	13.8	33.9

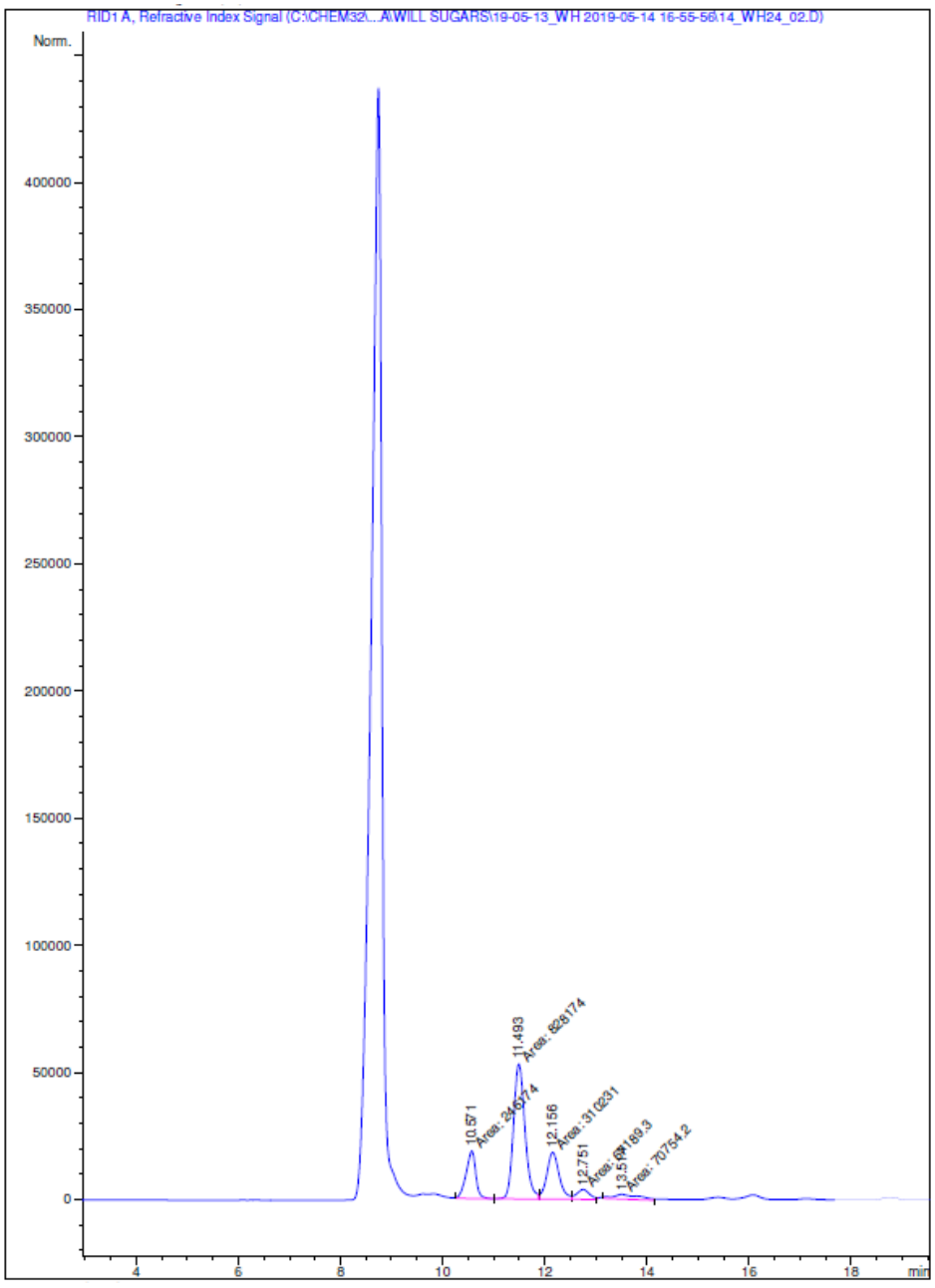


Figure 10: Chromatogram example from a temperature-assisted acid hydrolysis sample on the Agilent Hi-Plex column

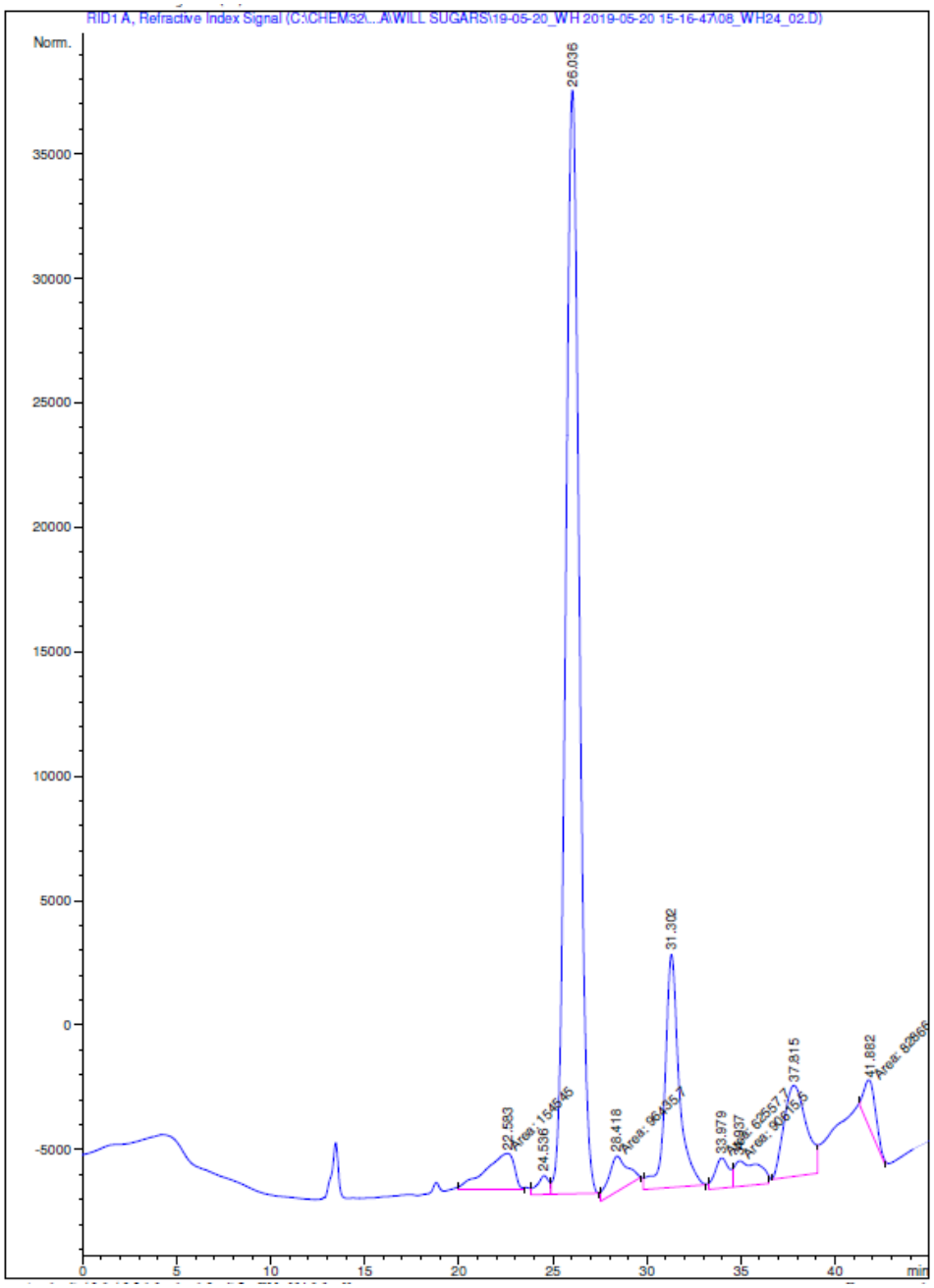


Figure 11: Chromatogram example from a temperature-assisted acid hydrolysis sample on the CARBOSep column

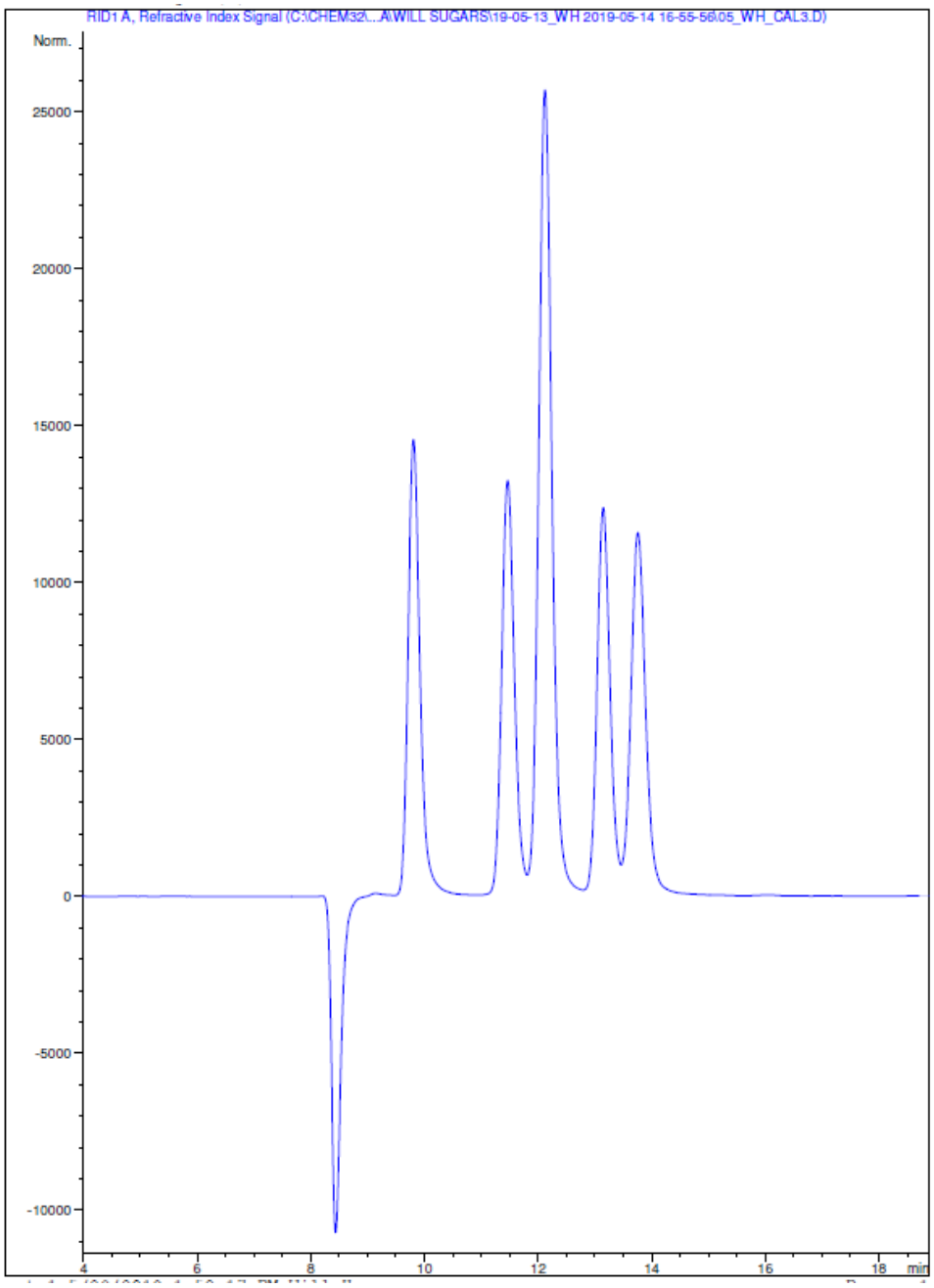


Figure 12: Calibration standard chromatogram for the Agilent Hi-Plex column

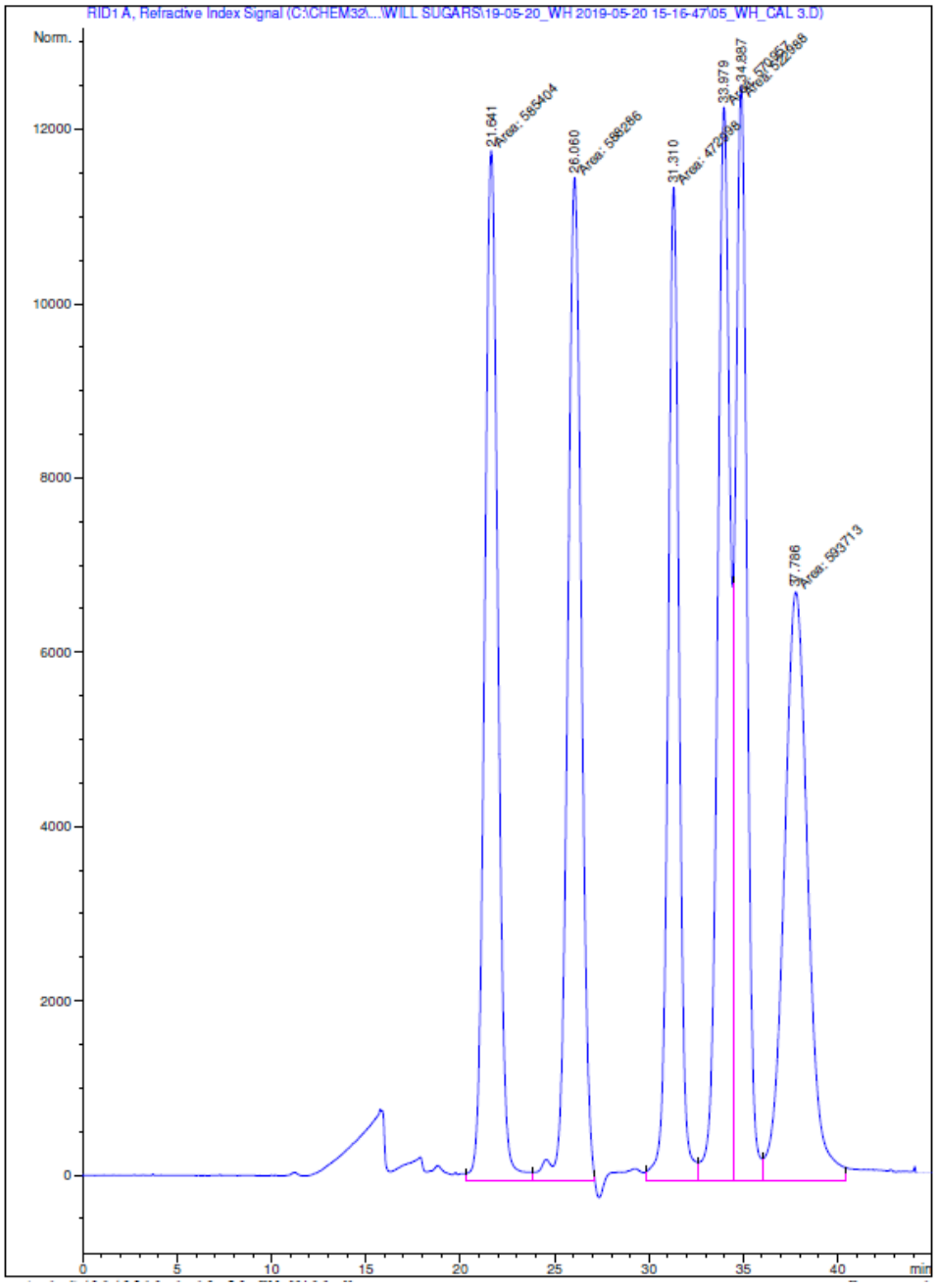


Figure 13: Calibration standard chromatogram for the CARBOSep column

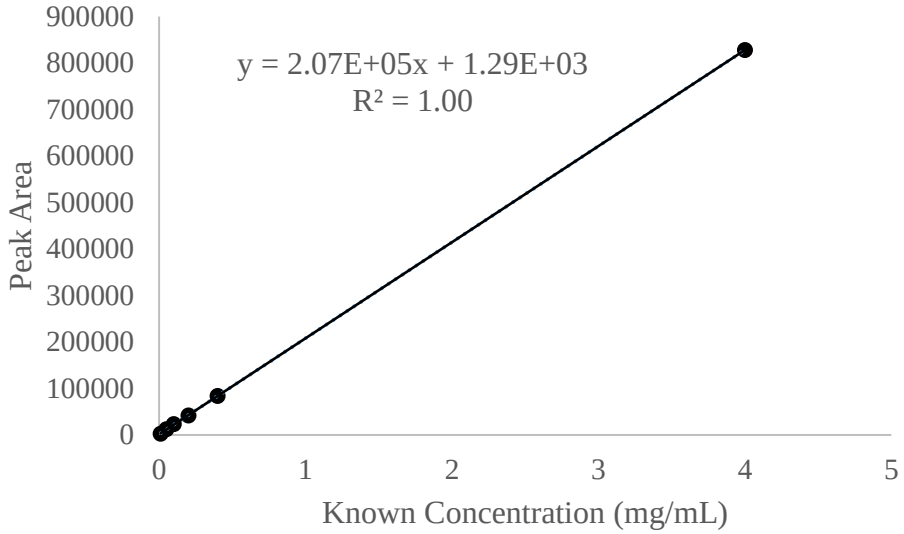


Figure 14: Calibration curve example showing the relation between known concentrations of sugar and HPLC response in area

The equation created from the calibration samples can be transformed into Equation 1 to calculate the sample concentration from the area given by the HPLC results. Concentrations are then manipulated to account for dilution factors and sugar recovery standards to find the actual concentration of sugar in the samples.

$$X = \frac{(Y - b)}{m} \qquad \text{Equation 1}$$

Where:

X = Sample concentration (mg/mL)
Y = Sugar area given by HPLC results
b = intercept from calibration equation
m = Slope from calibration equation

APPENDIX E

ALL DATA

Table 10: Complete design matrix for optimization study with results

Std Order	Run Order	Pt Type	Blocks	AC	HT	STLL	Carbohydrates Recovered (wt%)
1	10	1	1	1.0	10	10	55.43
2	60	1	1	4.0	10	10	40.60
3	29	1	1	1.0	20	10	36.37
4	51	1	1	4.0	20	10	66.40
5	42	1	1	1.0	10	20	52.60
6	18	1	1	4.0	10	20	64.47
7	32	1	1	1.0	20	20	54.80
8	49	1	1	4.0	20	20	74.55
9	50	-1	1	0.0	15	15	0.97
10	31	-1	1	5.0	15	15	63.28
11	40	-1	1	2.5	7	15	54.57
12	44	-1	1	2.5	23	15	51.58
13	24	-1	1	2.5	15	7	48.46
14	30	-1	1	2.5	15	23	51.64
15	34	0	1	2.5	15	15	48.45
16	11	0	1	2.5	15	15	58.44
17	57	0	1	2.5	15	15	54.03
18	55	0	1	2.5	15	15	53.07
19	21	0	1	2.5	15	15	51.50
20	9	0	1	2.5	15	15	57.27
21	52	1	1	1.0	10	10	36.45
22	45	1	1	4.0	10	10	60.14
23	1	1	1	1.0	20	10	44.92
24	4	1	1	4.0	20	10	81.57
25	47	1	1	1.0	10	20	56.90
26	43	1	1	4.0	10	20	56.18
27	23	1	1	1.0	20	20	57.12
28	33	1	1	4.0	20	20	85.72

Table 10: Continued

29	41	-1	1	0.0	15	15	0.99
30	46	-1	1	5.0	15	15	51.51
31	15	-1	1	2.5	7	15	57.69
32	59	-1	1	2.5	23	15	53.70
33	56	-1	1	2.5	15	7	53.31
34	14	-1	1	2.5	15	23	59.27
35	13	0	1	2.5	15	15	57.05
36	36	0	1	2.5	15	15	52.66
37	27	0	1	2.5	15	15	54.93
38	54	0	1	2.5	15	15	53.30
39	53	0	1	2.5	15	15	52.56
40	19	0	1	2.5	15	15	53.96
41	22	1	1	1.0	10	10	58.10
42	3	1	1	4.0	10	10	27.78
43	48	1	1	1.0	20	10	35.49
44	7	1	1	4.0	20	10	78.95
45	39	1	1	1.0	10	20	55.25
46	2	1	1	4.0	10	20	65.20
47	8	1	1	1.0	20	20	55.16
48	38	1	1	4.0	20	20	79.20
49	58	-1	1	0.0	15	15	1.30
50	5	-1	1	5.0	15	15	46.69
51	20	-1	1	2.5	7	15	53.19
52	35	-1	1	2.5	23	15	53.38
53	6	-1	1	2.5	15	7	49.89
54	26	-1	1	2.5	15	23	50.79
55	12	0	1	2.5	15	15	56.73
56	25	0	1	2.5	15	15	56.61
57	37	0	1	2.5	15	15	52.01
58	28	0	1	2.5	15	15	49.96
59	17	0	1	2.5	15	15	58.56
60	16	0	1	2.5	15	15	53.55

Table 11: HPLC area results for microwave optimization study

Sample	Glucose	Galactose	Total Carbs
7-1	66570.60	22900.67	90028.42
9-3	38747.27	13480.65	55675.36
7-2	101231.12	44548.74	184456.39
11-1	49221.73	13399.87	62621.60
11-2	108766.94	37055.90	151891.04
7-6	97996.21	43326.79	178581.02
7-4	41356.59	13643.28	55510.40
11-3	55300.04	18527.57	76605.31
9-2	75228.77	30077.79	111313.09
11-4	55953.85	17576.37	78183.94
11-5	55625.34	17078.34	75812.31
11-6	56318.59	18367.38	76513.69
11-7	36797.99	12075.12	50820.94
12-1	54143.67	18730.60	77158.76
11-8	53698.73	17421.08	71834.38
11-9	55583.87	18187.72	78530.28
9-8	61315.78	27454.21	84998.75
11-10	54004.05	16955.80	72228.71
12-2	52525.54	17143.96	71136.44
11-11	51654.34	16756.08	68944.29
7-7	40411.76	14118.58	57342.01
11-12	109978.69	36139.81	147652.75
11-13	54567.27	18361.98	75627.35
11-14	31591.68	10382.24	43572.68
11-15	53428.38	17066.06	73495.43
11-16	50395.02	16455.75	66880.33
7-5	52426.75	18561.49	72991.68
11-17	33687.69	10219.31	44292.53
11-18	57743.98	20119.54	84858.71
7-10	40644.87	13410.95	54992.74
7-3	65345.47	32298.71	97952.18
11-19	49189.93	14855.85	64783.35
12-4	50153.66	17039.49	71607.57

Table 11: Continued

11-20	52842.90	16298.82	70387.59
11-21	51002.40	15562.26	69677.35
9-1	40386.86	14185.08	55505.25
12-3	53604.47	17341.12	72906.51
11-22	700.75	528.68	1332.23
9-9	39447.33	15337.89	56385.58
12-5	51272.13	16632.42	69101.09
9-7	87785.37	30152.33	120647.79
11-23	47411.75	15612.42	68921.77
7-8	35535.59	13833.68	51247.76
7-11	45168.04	19544.77	64815.62
11-24	822.71	452.03	1298.31
12-7	52175.07	17062.37	70374.01
12-9	52849.99	16843.58	71149.55
12-10	114359.82	38834.72	162567.79
12-11	53576.80	17060.85	72233.89
12-6	52176.78	17605.76	71851.66
9-11	52697.11	18985.22	84522.39
14-1	106411.40	36114.12	146032.48
14-2	72790.92	27471.01	107327.87
14-3	57361.89	22987.83	83877.47
14-4	135079.58	41896.06	182269.65
14-5	152195.36	50123.37	210096.63
14-6	90079.94	30893.06	145009.62
14-7	79209.31	26822.30	106031.60
14-8	80934.60	30439.28	112083.85
14-9	86787.15	36951.97	150636.66
14-10	78030.90	24334.87	106061.34
14-11	87725.26	27509.78	117044.35
14-12	78728.08	24343.36	106283.97
15-1	117403.11	34525.39	152467.03
15-2	127328.93	37866.61	165884.29
15-3	125559.02	36827.29	163027.41
15-4	124225.18	37667.32	162517.08
15-5	113125.07	32431.60	145986.84

Table 11: Continued

15-6	126994.85	38172.13	165827.39
15-7	62165.47	19419.06	81868.62
15-8	65251.04	19953.54	88637.51
15-9	63039.31	18437.39	85015.85
15-10	42719.53	11600.77	54445.49
15-11	55248.02	18585.81	77473.28
15-12	49362.56	14427.49	64147.88
16-1	143327.25	50515.91	206402.65
16-2	157086.56	52847.62	218693.14
16-3	151864.05	53264.98	213000.00
18-4	104257.56	42186.84	147040.23
18-5	85388.77	30119.72	115979.26
18-6	105733.93	43172.16	149264.64
18-7	73334.43	22372.08	97042.28
18-8	36963.20	65107.36	102885.74
18-9	39426.29	71069.22	111158.35
18-10	2069.36	723.53	2944.55
18-11	24881.40	24910.45	59983.71
18-12	474.51	540.56	1196.85
19-1	115597.44	40092.69	159146.08
19-2	112190.24	38226.57	153310.55
19-4	65109.64	30523.08	95632.72
19-5	8531.83	621.51	9153.33
19-6	3882.46	1694.84	5577.30

Table 12: HPLC calibrations for total carbohydrate test from 11/28/2018

Sample	Glucose (mg/mL)	Galactose (mg/mL)	Mannose (mg/mL)	Galactose + Mannose (mg/mL)	Arabinose (mg/mL)
Cal 1	0.1	0.1	0.1	0.2	0.1
Cal 2	0.5	0.5	0.5	1	0.5
Cal 3	1	1	1	2	1
Cal 4	2	2	2	4	2
Cal 5	4	4	4	8	4

Table 13: HPLC results for calibrations of total carbohydrate test from 11/28/2018

Sample	Glucose Area	Galactose + Mannose Area	Arabinose Area
Cal 1	37110.2	74650.4	35869.35
Cal 2	180183	364219.85	164432.1
Cal 3	362063	731006.5	329977.3
Cal 4	3190612	1421132.6	642531.7
Cal 5	1448619	2909653.7	1337620

Table 14: Sugar recovery standards for total carbohydrate test from 11/28/2018

Sample	Start (mg/mL)	LC Area	After (mg/mL)	% Recovery
Glu1	1.22	247668	0.98534	80.77
Glu2	1.0002	272744	1.00218	100.20
Glu3	1.0002	270706	1.00081	100.06
Gal1	1.01	261525	0.72774	72.05
Gal2	1.0012	249326	0.6941	69.33
Gal3	1.0012	256021	0.71256	71.17
Ara1	0.9977	260791	0.79634	79.82
Ara2	0.9977	261906	0.79969	80.15
Ara3	0.9977	252252	0.77074	77.25

Table 15: HPLC results for total carbohydrate test for non-milled biomass from 11/28/2018

Sample	Glucose Area	Galactose + Mannose Area	Arabinose Area
1.0	145945	52742.8	8478
2.0	150529	62144	9691.4
3.0	151886	66018.2	11912.6
4.0	155678	64014.1	8674.4
4.1	194477	71109.3	11809.6
1.0	287755	119886	23704
2.0	282217	136619	41981

Table 16: Total carbohydrate test sugar weight results for non-milled biomass from 11/28/2018

Sample	Sample wt (mg)	Act wt (mg)	Glucose wt (mg)	Galactose + Mannose wt (mg)	Arabinose wt (mg)
1	289.9	266.33	85.17	18.67	4.36
2	303.6	278.92	85.45	21.86	4.76
3	297.4	273.22	85.54	23.17	5.50
4	304.2	279.47	85.77	22.49	4.43
4.1	304.2	279.47	88.19	24.89	5.46
1.1	297.8	273.59	94.01	29.34	9.39
2.1	300.5	276.07	93.67	33.35	15.42

Table 17: HPLC results for total carbohydrate test for milled biomass from 11/28/2018

Sample	Glucose Area	Galactose + Mannose Area	Arabinose Area
2.0	151510.2	65727.1	12772.2
3.0	176158.3	75988	15521.8
4.0	169088.7	75585.9	20646.4
4.1	217384.1	74302.8	12063.3
1.0	163425.4	61353.7	6423.6
2.1	141486.2	65144.7	4372.7

Table 18: Total carbohydrate test sugar weight results for milled biomass from 11/28/2018

Sample	Sample wt (mg)	Act wt (mg)	Glucose wt (mg)	Galactose + Mannose wt (mg)	Arabinose wt (mg)
2	298.2	273.96	80.11	16.34	4.57
3	300.2	275.80	81.55	18.81	5.29
4	298.7	274.42	81.13	18.71	6.62
4.1	298.7	274.42	83.95	18.40	4.38
1	296.1	272.03	86.26	21.59	3.68
2.1	296.1	272.03	84.89	22.87	3.01

Table 19: HPLC calibration standards for sonication study from 04/02/2019

Sample	Glucose (mg/mL)	Galactose (mg/mL)	Mannose (mg/mL)	Galactose + Mannose (mg/mL)	Arabinose (mg/mL)	Fucose (mg/mL)
Cal 1	0.01	0.01	0.01	0.01	0.01	0.01
Cal 2	0.05	0.05	0.05	0.1	0.05	0.05
Cal 3	0.1	0.1	0.1	0.2	0.1	0.1
Cal 4	0.2	0.2	0.2	0.4	0.2	0.2
Cal 5	0.4	0.4	0.4	0.8	0.4	0.4
Cal 6	4	4	4	8	4	4

Table 20: HPLC results for calibration standards from 04/02/2019

Sample	Glucose Area	Galactose + Mannose Area	Arabinose Area	Fucose Area
Cal 1	2425.9	4605.4	1638.6	6139
Cal 2	12345.9	21528.4	10662.5	13651.6
Cal 3	23323.9	43100	20679	24747.4
Cal 4	41761.1	85618.2	41924.1	44528
Cal 5	83608.6	168608.3	82742.2	86568.7
Cal 6	828038	1694728.4	837771.4	883886

Table 21: HPLC results of sonication study for samples from 04/02/2019

Sample	Glucose Area	Galactose + Mannose Area	Arabinose Area	Fucose Area	Unknown Sugar Area
22-10	664417	260805	35406.9	31620.6	301963.1
22-11	701466	276441.8	39925.8	39854.3	320774.6
22-12	742920	290960.2	38916.6	44073.2	316924.9
22-13	739420	290053.7	38862.8	45736.9	314749.6
22-14	641659	253280.6	36788.8	39161.6	296574.7
22-15	704886	278275.3	40212.8	42459.9	324766.2
22-16	759719	297249.3	39035.2	44557.7	324326.5
22-17	768311	301139.2	38370.2	44101.5	330923.6
22-18	580476	230393.6	27698.9	36695.9	293727.8
22-19	681347	267514.4	29745.3	43009.1	315841.3

Table 22: Sonication sugar weight results for samples from 04/02/2019

Sample	Sample wt (mg)	Act wt (mg)	Glucose wt (mg)	Galactose + Mannose wt (mg)	Arabinose wt (mg)	Fucose wt (mg)	Unknown Sugar wt (mg)
22-10	509.9	468.4	68.51	34.67	4.31	3.06	28.97
22-11	507.7	466.4	72.33	36.75	4.86	3.47	30.79
22-12	508.3	467.0	76.62	38.69	4.74	3.38	30.42
22-13	499.5	458.9	76.25	38.57	4.73	3.37	30.21
22-14	509.5	468.1	66.16	33.67	4.48	3.18	28.45
22-15	500.4	459.7	72.69	37.00	4.89	3.50	31.18
22-16	509.7	468.3	78.35	39.53	4.75	3.39	31.14
22-17	504.2	463.2	79.24	40.05	4.67	3.33	31.77
22-18	509.9	468.4	63.88	43.21	4.28	2.36	28.17
22-19	508.3	467.0	75.00	50.19	4.59	2.55	30.31

Table 23: HPLC calibration standards for autoclave time study from 05/14/2019

Sample	Glucose (mg/mL)	Galactose (mg/mL)	Mannose (mg/mL)	Galactose + Mannose (mg/mL)	Arabinose (mg/mL)	Fucose (mg/mL)
Cal 1	0.1	0.1	0.1	0.02	0.1	0.1
Cal 2	0.5	0.5	0.5	1	0.5	0.5
Cal 3	1	1	1	2	1	1
Cal 4	4	4	4	8	4	4

Table 24: HPLC results for calibration standards from 05/14/2019

Sample	Glucose Area	Galactose + Mannose Area	Arabinose Area	Fucose Area
Cal 1	21858.1	43624.6	20550.6	23742.3
Cal 2	110285.3	216963.5	102246.3	108666.7
Cal 3	208829.1	419645.3	195983.1	205407.4
Cal 4	852580.1	1712253.6	807634.8	844786.6

Table 25: HPLC results of autoclave time study for samples from 05/14/2019

Sample	Glucose Area	Galactose + Mannose Area	Arabinose Area	Unknown Sugar Area
23-01	747922	285404.7	80279.1	302263.7
23-02	766436.4	268159.9	66067.8	263268.5
23-03	768278.5	278032.7	68687.6	293138
23-04	764576.4	289114.2	77942.1	292712.1
23-05	754139.5	271855.5	68289.5	288203.7
23-06	762478.6	291511.3	63725.1	290448.8
24-01	892301.9	331289.1	65910.1	344200.4
24-02	828174.3	310231.4	70754.2	313363.5
24-03	880528.1	334114.1	84264.8	353846.9
24-07	821501.1	314978.3	74326.6	343756.6
24-08	757286.6	300492.4	77619.4	325702.1
24-09	735949.2	268273.4	56728.7	290934
25-01	813465.3	291518.3	46002.1	301898.3
25-02	828017.3	313208.4	88943.1	337506.8
25-03	824303.1	340776.2	71676.1	344044.6

Table 26: Autoclave time study sugar weight results for samples from 05/14/2019

Sample	Sample wt (mg)	Act wt (mg)	Glucose wt (mg)	Galactose + Mannose wt (mg)	Arabinose wt (mg)	Unknown Sugar wt (mg)
23-01	495.2	454.9	74.97	37.79	10.20	28.34
23-02	496.9	456.5	76.83	35.52	8.42	24.67
23-03	502	461.2	77.01	36.82	8.75	27.48
23-04	506.5	465.3	76.64	38.28	9.91	27.44
23-05	499.2	458.6	75.60	36.01	8.70	27.01
23-06	505.9	464.8	76.43	38.60	8.13	27.23
24-01	502.8	461.9	88.97	42.45	8.40	32.27
24-02	508.6	467.3	82.58	39.76	9.01	29.38
24-03	511.6	470.0	87.80	42.81	10.70	33.18
24-07	500	459.4	82.35	41.69	9.46	32.23
24-08	498.1	457.6	75.91	39.78	9.87	30.54
24-09	498.5	458.0	73.77	35.53	7.25	27.27
25-01	502	461.2	81.11	37.37	5.91	28.30
25-02	495.4	455.1	82.56	40.14	11.29	31.65
25-03	503	462.1	82.19	43.66	9.13	32.26

Table 27: HPLC calibration standards for sugar column study from 05/21/2019

Sample	Glucose (mg/mL)	Galactose (mg/mL)	Mannose (mg/mL)	Arabinose (mg/mL)	Fucose (mg/mL)
Cal 1	0.1	0.1	0.1	0.1	0.1
Cal 2	0.5	0.5	0.5	0.5	0.5
Cal 3	1	1	1	1	1
Cal 4	4	4	4	4	4

Table 28: HPLC results for calibration standards from 05/21/2019

Sample	Glucose Area	Galactose Area	Mannose Area	Arabinose Area	Fucose Area
Cal 1	55462.2	43525	54246.6	50788.8	53706.3
Cal 2	289373	232922.2	285822.8	262816.3	282738.8
Cal 3	588286	472998.3	593713.3	522988.4	570956.6
Cal 4	2281320	1858537.3	2246351.2	20199918	2240149

Table 29: HPLC results of sugar column study for samples from 05/14/2019

Sample	Glucose Area	Galactose Area	Mannose Area	Arabinose Area	Fucose Area	Unknown Sugar Area
24-01	2379569	520462.5	283080.3	81019.8	90155.1	383166
24-02	2233306	485831.1	304052.2	87335.3	82557.7	333600.2
24-03	2394728	479349.2	302592	90615.5	89094	369042.7

Table 30: Sugar column study sugar weight results for samples from 05/14/2019

Sample	Sample wt (mg)	Glucose wt (mg)	Galactose wt (mg)	Mannose wt (mg)	Arabin wt (mg)	Fucose wt (mg)	Unknown Sugar wt (mg)
24-01	461.9	88.51	24.67	12.29	9.91	3.92	12.78
24-02	467.3	83.05	23.01	13.24	9.93	3.58	11.04
24-03	470.0	89.07	22.70	13.17	9.94	3.87	12.29

Table 31: Sugar recovery standards study for 30-minute autoclave from 05/21/2019

Sample	Glucose Start (mg/mL)	Galactose Start (mg/mL)	Glucose After (mg/mL)	Galactose After (mg/mL)	Glucose % Recovery	Galactose % Recovery
25-4	2.23	2.34	2.19	1.69	93.5	72.2
25-5	2.19	2.39	2.27	1.77	94.8	74.2

Table 32: Oil recovery temperature study from 5/29 through 6/5/2019

Sample	Temperature (°C)	Oil wt% of Biomass
27-01	200	19.9
27-02	200	17.2
27-03	140	19.9
27-04	140	19.3
27-05	120	16.4
27-06	120	16.3
28-01	180	21.8
28-02	180	20.7
28-03	180	24.1

Table 33: HPLC calibration standards for analysis of carbohydrates recovered from lipid extracted biomass on 5/29 through 6/6/2019

Sample	Glucose (mg/mL)	Galactose (mg/mL)	Mannose (mg/mL)	Galactose + Mannose (mg/mL)	Arabinose (mg/mL)	Fucose (mg/mL)
Cal 1	0.1	0.1	0.1	0.02	0.1	0.1
Cal 2	0.5	0.5	0.5	1	0.5	0.5
Cal 3	1	1	1	2	1	1
Cal 4	4	4	4	8	4	4

Table 34: HPLC Results of calibrations from 5/29/2019

Sample	Glucose Area	Galactose + Mannose Area	Arabinose Area	Fucose Area
Cal 1	21032.6	40776.1	20271.4	22680.9
Cal 2	109954	216999.8	101969.7	106270.3
Cal 3	211151	423416.9	198760.8	206808.7
Cal 4	849363	1705166.6	801181.7	817817.8

Table 35: HPLC results from carbohydrate recovery of lipid extracted biomass on 5/29 through 6/6/2019

Sample	Glucose Area	Galactose + Mannose Area	Arabinose Area	Fucose Area	Unknown Sugar Area
27-01	948703	282771.5	51718.2	38289.6	388392.3
27-02	989433	297091.4	54096.5	39200.8	403240.9
27-03	1035317	305852.8	56312.1	40325.1	407982
27-04	985524	291646.8	54390.7	39648.7	389196.2
27-05	972750	294100.4	54331.1	44279.2	401238.9
27-06	934348	281294.2	52159.6	37931.8	381332
28-01	825308	223940.2	41491.2	21408.4	308398
28-02	824696	242924.6	41447.9	31319.9	355685.8
28-03	862121	257647.2	34466.3	21818.5	361616.6
29-04	151367	48511.2	9517	6510.2	75245.2
29-05	147454	47786.7	8771.2	3559.5	75998.2
29-06	143982	47487.6	8608.8	7630.6	66747.5
30-04	1379485	420287.8	74918.7	56060.7	598823.1
30-05	1346030	412007.4	73549.7	60582.7	582462.3
30-08	14074.1	13197.8	-	8408.5	-
30-09	13372.2	13857.6	-	11264.9	-

Table 36: Carbohydrate weight results from lipid extracted biomass on 5/29 through 6/6/2019

Sample	Sample wt (g)	Glucose wt (mg)	Galactose + Mannose wt (mg)	Arabinose wt (mg)	Fucose wt (mg)	Unknown Sugar wt (mg)
27-01	0.715	113.0	49.5	18.4	8.4	46.9
27-02	0.738	121.5	53.3	19.1	8.8	50.1
27-03	0.664	114.3	49.2	17.2	8.1	45.6
27-04	0.655	107.5	46.6	16.9	7.9	43.1
27-05	0.740	119.9	53.0	19.1	9.6	50.1
27-06	0.727	113.1	50.1	18.8	8.5	46.9
28-01	0.721	124.1	42.8	7.7	3.4	42.0
28-02	0.705	116.5	43.7	7.2	4.9	45.8
28-03	0.741	128.1	48.7	6.3	3.5	49.0
29-04	0.308	65.3	27.1	5.0	1.7	30.0
29-05	0.309	63.6	26.7	4.6	0.2	30.3
29-06	0.304	62.1	26.5	4.5	2.3	26.5
30-04	0.778	164.8	65.0	11.1	7.6	66.9
30-05	0.774	160.8	63.7	10.9	8.3	65.0
30-08	0.323	4.9	6.0	-	1.4	-
30-09	0.295	4.6	6.4	-	2.9	-

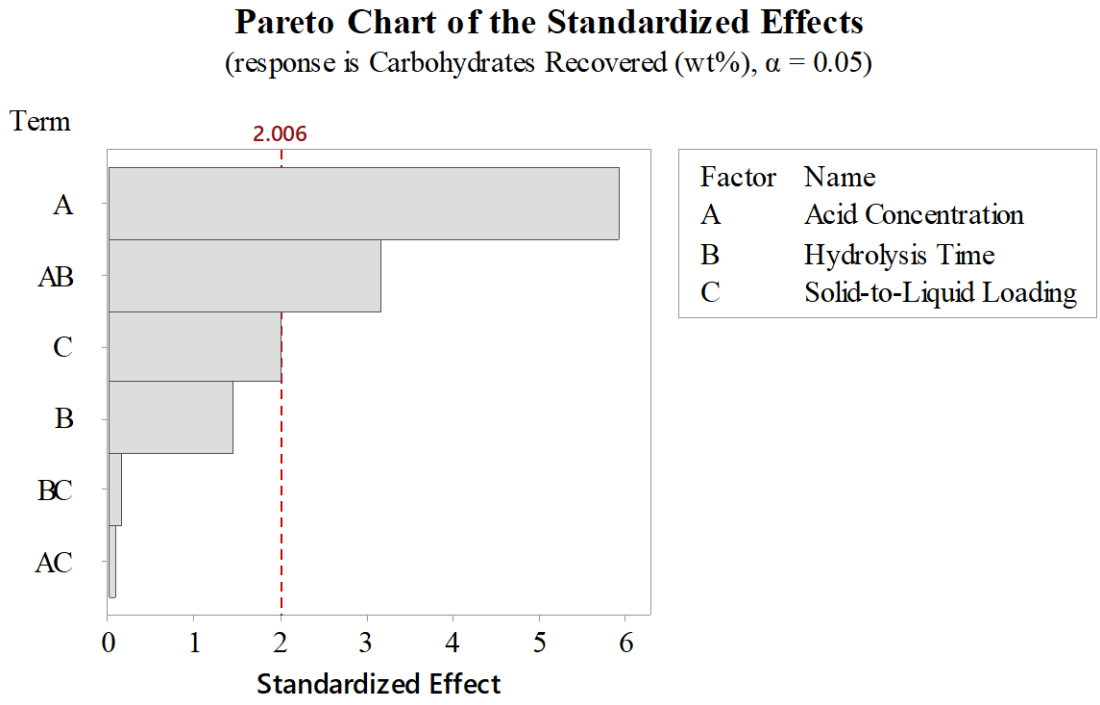
APPENDIX F

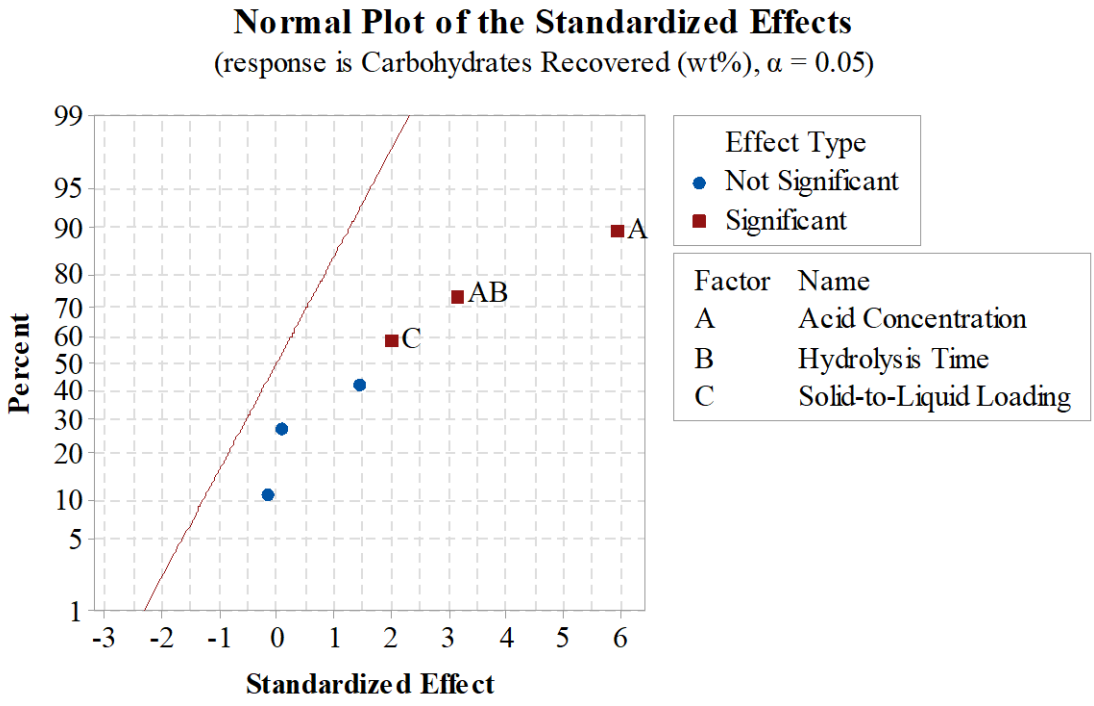
MINITAB ANOVA AND PLOTS

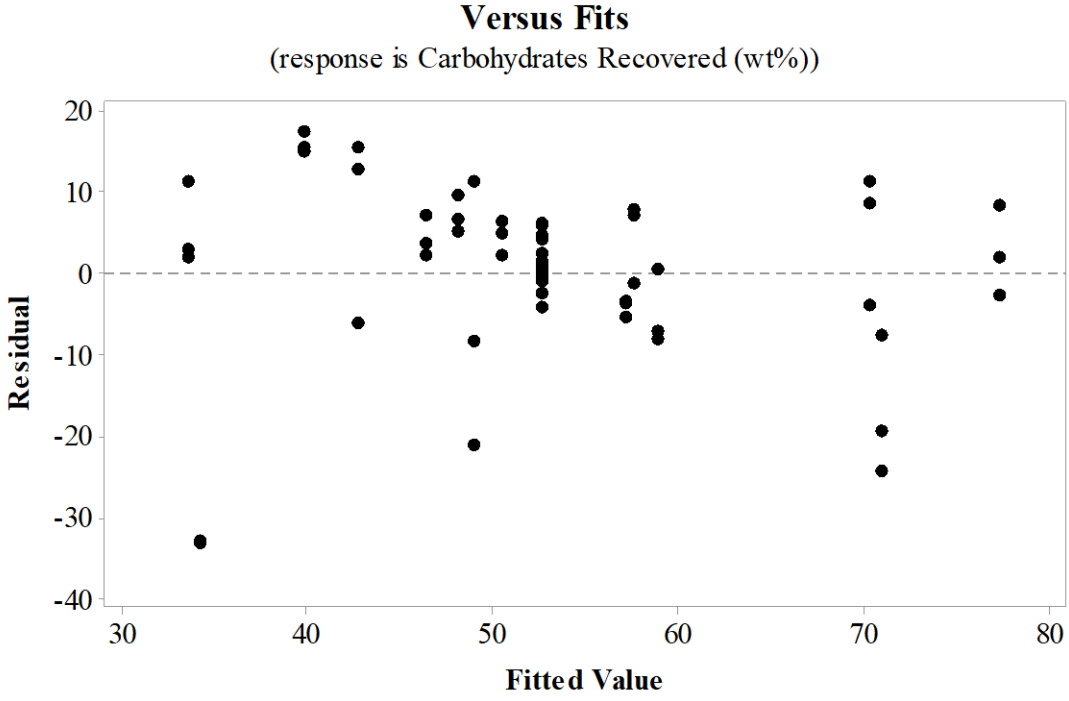
In order to determine the significant variables for total carbohydrate recovery, a Pareto chart was constructed. The chart showed the effect of the independent variables and their interactions on total carbohydrate recovery. The t-ratio in the chart was compared to a critical t value which was shown as the vertical red line in the Pareto chart. The independent variables who's chart length passed the red vertical line were significant factors of carbohydrate recovery from microalgae. Since the chart length of the solid-to-liquid loading factor is on the line, a normal plot was generated in order to determine its significance.

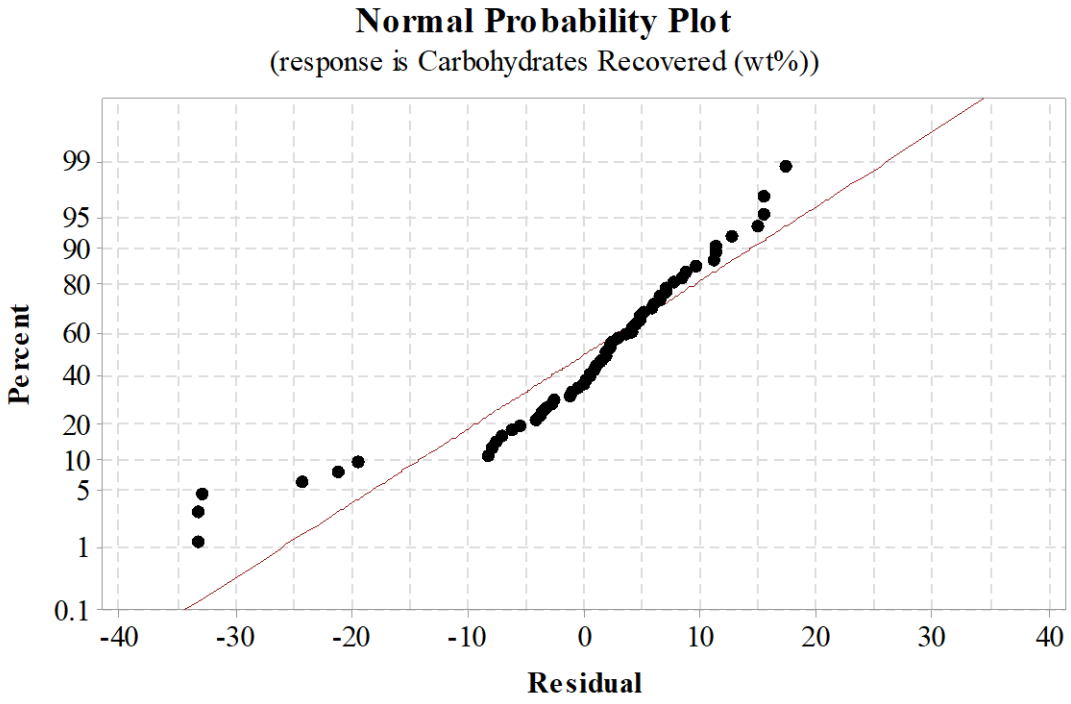
Versus fits plot is used to determine the scatter of the data and if there is a trend that occurred from the run order. The normal probability plot is used to determine outliers and if the data is normally distributed. Since there is no clear trend in the versus fits and the normal probability plot is a straight line with no clear outliers, we can confirm that there are no clear trends from the run order. The interaction plot works similar to the contour plot to determine if the optimum conditions based on the interactions between factors. The following figures are individually labeled with their respective chart title. Also included is the ANOVA table which is used to see P-values of each factor and interaction to further solidify significance. If there the factor/interaction has a P-value

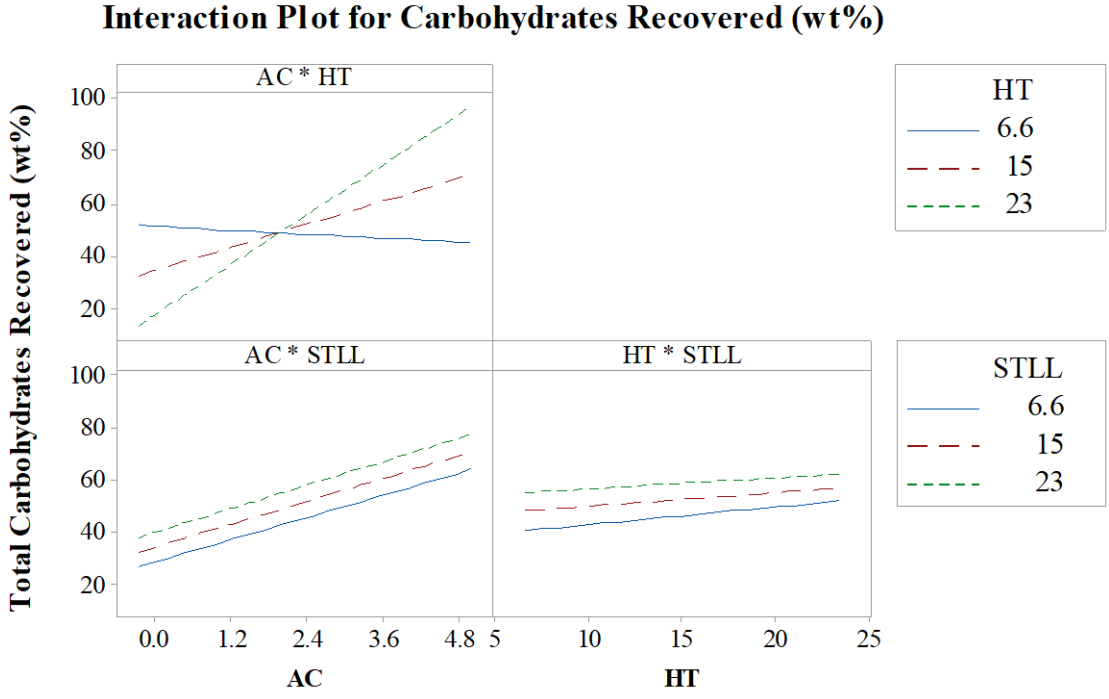
less than 0.05, it is considered significant. The ANOVA table also includes regression equation that will allow to determine the theoretical carbohydrate recovery.




Normal Plot of the Standardized Effects
(response is Carbohydrates Recovered (wt%), α = 0.05)
Percent
99
95
90
80
70
60
50
40
30
20
10
5
1
-3
-2
-1
0
1
2
3
4
5
6
Standardized Effect
A
AB
C
Effect Type
Not Significant
Significant
Factor Name
A Acid Concentration
B Hydrolysis Time
C Solid-to-Liquid Loading


Versus Fits
(response is Carbohydrates Recovered (wt%))
Residual
20
10
0
-10
-20
-30
-40
30
40
50
60
70
80
Fitted Value


Normal Probability Plot
(response is Carbohydrates Recovered (wt%))
Percent
99
95
90
80
60
40
20
10
5
1
0.1
-40
-30
-20
-10
0
10
20
30
40
Residual


Interaction Plot for Carbohydrates Recovered (wt%)
AC * HT
AC * STLL
HT * STLL
Total Carbohydrates Recovered (wt%)
100
80
60
40
20
0.0
1.2
2.4
3.6
4.8
5
10
15
20
25
AC
HT
HT
6.6
15
23
STLL
6.6
15
23

Response Surface Regression: Carbohydrates Recovered ... Time, Ratio

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	9	9835.9	1092.88	11.69	0.000
Linear	3	5761.2	1920.38	20.54	0.000
Concentration	1	4904.3	4904.34	52.45	0.000
Time	1	293.9	293.88	3.14	0.082
Ratio	1	562.9	562.94	6.02	0.018
Square	3	2676.2	892.08	9.54	0.000
Concentration*Concentration	1	1877.7	1877.69	20.08	0.000
Time*Time	1	339.1	339.10	3.63	0.063
Ratio*Ratio	1	203.1	203.13	2.17	0.147
2-Way Interaction	3	1398.5	466.17	4.99	0.004
Concentration*Time	1	1394.0	1393.97	14.91	0.000
Concentration*Ratio	1	1.0	0.96	0.01	0.920
Time*Ratio	1	3.6	3.56	0.04	0.846
Error	50	4674.9	93.50		
Lack-of-Fit	5	3200.5	640.09	19.54	0.000
Pure Error	45	1474.4	32.76		
Total	59	14510.8			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
9.66941	67.78%	61.98%	48.28%

Coded Coefficients

Term	Coef	SE Coef	T-Value	P-Value	VIF
Constant	53.71	2.28	23.59	0.000	
Concentration	18.40	2.54	7.24	0.000	1.00
Time	4.50	2.54	1.77	0.082	1.00
Ratio	6.23	2.54	2.45	0.018	1.00
Concentration*Concentration	-18.64	4.16	-4.48	0.000	1.02
Time*Time	7.92	4.16	1.90	0.063	1.02
Ratio*Ratio	6.13	4.16	1.47	0.147	1.02

Concentration*Time	21.56	5.58	3.86	0.000	1.00
Concentration*Ratio	0.57	5.58	0.10	0.920	1.00
Time*Ratio	-1.09	5.58	-0.20	0.846	1.00

Regression Equation in Uncoded Units

Carbohydrates Recovered (wt%)	=	78.4 + 6.30 Concentration - 5.13 Time - 1.70 Ratio - 2.929 Concentration*Concentration + 0.1120 Time*Time + 0.0867 Ratio*Ratio + 1.016 Concentration*Time + 0.027 Concentration*Ratio - 0.0154 Time*Ratio

Fits and Diagnostics for Unusual Observations

Obs	Carbohydrates Recovered (wt%)	Fit	Resid	Std Resid	
27	57.12	39.33	17.80	2.09	R
42	27.78	48.44	-20.66	-2.42	R

R Large residual

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